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Factors affecting cholinesterase activity in aquatic animals

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Factors affecting cholinesterase activity in aquatic animals

by

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For the Graduate College

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GENERAL INTRODUCTION

Organophosphorus insecticides (OPs) are extensively used throughout the world to control crop pests (Eto, 1991; ERS, 1994). As organochlorine insecticides have gradually been phased out in response to problems caused by their persistence in the environment, OPs have been a common choice to replace them (Ware, 1994). In the United States, for example, the top three insecticides applied to field crops in 1993 were chlorpyrifos (3×10^6 kg), terbufos (2.5×10^6 kg), and methyl parathion (2.3×10^6 kg; ERS, 1994).

OPs can enter aquatic systems that drain agricultural watersheds through routes such as direct application for aquatic insect pest control (Yasuno, 1991; Stevens and Warren, 1992), aerial overspray (Hennessey et al., 1992), and runoff from crop fields (Cooper, 1991; Richards and Baker, 1993; Dieter et al., 1995). Because insecticides that target aquatic pests are formulated for greater effectiveness in water, direct application can result in higher concentrations (e.g., up to 10 μg/L; Yasuno, 1991), and relatively longer-term exposures (e.g., 2-6 weeks; Yasuno, 1991) than those resulting from other uses. Runoff from fields occurs with rain, and like aerial overspray, results in pulsed events with relatively short exposure periods.

Assessment of an aquatic system's exposure to OPs is difficult as these compounds are non-persistent (Ware, 1994); they are rapidly hydrolyzed and photolyzed (Weinberger et al., 1982; Wang et al., 1989), taken up by biota (Brock et al., 1992), and they readily adsorb to sediments (Sharom et al., 1980; Knuth and

Heinis, 1992). Water solubilities of many OPs are low; for example, the solubility of chlorpyrifos is 1-2 mg/L at 25°C, and its mean soil adsorption coefficient (K_d) is 173 mL/g (Racke, 1993). Therefore, OPs may be found in the aquatic ecosystem for only short intervals following rain events, adsorbed to particulates in runoff. Timing of sample collections and expense of analyses make direct monitoring of these insecticides problematic.

Because OPs are difficult and expensive to monitor in the field, a reliable indicator of exposure and resulting toxic effects is needed. Such an indicator must integrate across exposure events to provide evidence of prior exposure at sampling times. The indicator must also be sensitive to OP exposure at concentrations present during exposure events. Finally, it must be less expensive to measure than analyses of insecticide residues in water or animal tissue.

Monitoring of exposure is important because OPs are highly toxic to aquatic organisms (Zinkl et al., 1991; Fairchild et al., 1992; Fisher et al., 1993; van Wijngaarden et al., 1996), and may substantially alter community structure (Yasuno, 1991). OPs are toxic to fish and aquatic invertebrates at low concentrations. In bluegill (Lepomis macrochirus), for example, the 96-hr LC_{50} is 2.4 μg/L for chlorpyrifos and 1.8 μg/L for terbufos (Mayer and Ellersieck, 1986). In larvae of the dipteran, Chironomus tentans, the 24-hr LC_{50} for chlorpyrifos is 3.7 μg/L (Karnak and Collins, 1974). Symptoms of acute poisoning in fish include muscle paralysis, hyperactivity and loss of equilibrium (Zinkl et al., 1991); morbid aquatic insects stop

spontaneous movements including ventilation, and do not move even when touched (Karnak and Collins, 1974; Siegfried, 1993).

OPs cause acute toxicity primarily through cholinesterase (ChE) inhibition (Matsumara, 1985). Cholinesterase is a group of enzymes present in all animals, which hydrolyzes the neurotransmitter, acetylcholine, as well as similar compounds (Silver, 1974). OP binding to ChE is largely irreversible due to formation of a covalent bond (Matsumara, 1985), and new enzyme synthesis is often required for recover from ChE inhibition. For this reason, ChE inhibition caused by OPs may persist for more than two to six weeks (van der Wel and Welling, 1989; Morgan et al., 1990; Carr et al., 1995). ChE inhibition in aquatic organisms has often been suggested as a way to demonstrate OP exposure (Weiss and Gakstatter, 1964; Varela and Augspurger, 1996). Carbamate insecticides also inhibit ChE, but carbamate binding to ChE is reversible over relatively short intervals (Szeto et al., 1985); however, ChE inhibition can be useful for monitoring exposure to carbamates if assayed early enough following exposure (Coppage, 1977). Because carbamates are used in lesser quantities than OPs, the majority of monitoring is concerned with OPs.

Cholinesterase activity compares favorably with chemical analysis of insecticide residues as an indicator of OP exposure. ChE inhibition often persists for longer intervals than insecticide residues are detectable in water (Brock et al., 1992; Knuth and Heinis, 1992). ChE inhibition is a sensitive exposure indicator of low OP concentrations: ChE activity is significantly reduced at OP concentrations as

low as 1 μg/L. For example, exposure for 24 hr to 1 μg/L fenitrothion caused 40% ChE inhibition in the amphipod, Gammarus pulex (Kuhn and Streit, 1994). Rainbow trout (Oncorhynchus mykiss) exposed to 1 μg/L methidathion for 96 hr had 23% ChE inhibition (Flammarion et al., 1996). ChE analysis is less expensive than residue analysis: the colorimetric method of Ellman et al. (1961), for example, uses a spectrophotometer, whereas residue analysis is generally done with gas chromatography or high performance liquid chromatography. ChE analysis also can be performed on crude tissue homogenates (Fairbrother et al., 1991), while residue analysis requires tissue extractions.

The major disadvantage of using ChE inhibition rather than residue analysis is that there is a high variability in ChE activity which confounds determination of whether enzyme activity is inhibited. There are differences between species, as well as different responses within a species to different ChE-inhibiting compounds (Habig and Di Giulio, 1991). Most serious, however, is the fact that ChE activity differs among individuals of the same species. Causes of this variation are not fully understood.

Potential sources of variation include differences in age, sex, reproductive status, and stressors such as water temperature, dissolved oxygen concentration, and exposure to multiple contaminants (Rattner and Fairbrother, 1991). ChE activity is often greater in younger, smaller organisms than in older, larger ones (Weiss, 1961; Rath and Misra, 1980; Zinkl et al., 1987; Gard and Hooper, 1993; Fishwick et al., 1996). Serum and whole body ChE activity has been shown to vary

by sex and reproductive status in birds and fish, though the direction of variation differs among species; such differences are attributed to endocrine influences (van der Wel and Welling, 1989; Rattner and Fairbrother, 1991). Brain ChE activity, on the other hand, has not differed by sex nor reproductive status in any vertebrate studied so far (Hogan, 1970; Coppage, 1972; Rattner and Fairbrother, 1991). In some studies, ChE activities have increased with increasing water temperature (Hazel, 1969; Hogan, 1970), while in others, water temperature did not appear to affect ChE activity (Zinkl et al., 1987; Cole, 1995). Low dissolved oxygen may affect ChE activity: exposure of rainbow trout (O. mykiss) to the OP, dichlorvos, in hypoxic water increased ChE inhibition beyond that caused by dichlorvos exposure alone (Hoy et al., 1991).

If the sources of individual variability in ChE activity can be understood, then such variability can be reduced in future monitoring programs. This study was done to investigate some of the major potential sources of variation, using a large data set from a monitoring project, as well as experiments involving locally collected bluegill (L. macrochirus) and lab-cultured chironomids (Chironomus riparius). Bluegill collected alongside cornfields in two growing seasons as part of a monitoring project in the Mark Twain National Wildlife Refuge were analyzed for ChE inhibition. This data set was used to attempt to determine whether water temperature, fish size or fish sex affected brain ChE activity. Locally captured bluegill were subjected to one of two methods of euthanasia, and lab-cultured chironomid larvae were exposed to chlorpyrifos in hypoxic conditions and in

mixtures with two herbicides, to determine effects on ChE activity. Results from this study will be used to provide recommendations for monitoring programs using ChE activity to monitor for OP exposure.

Dissertation organization

This dissertation contains two chapters, each consisting of a paper to be submitted to an appropriate journal. Chapter 1 will be submitted to Aquatic Toxicology, with the following authorship: Sheryl L. Beauvais, Gary J. Atchison and Michael Coffey. Chapter 2 will be submitted to Hydrobiologia, with the following authorship: Sheryl L. Beauvais, Gary J. Atchison, Jana Stenback and William G. Crumpton. Each chapter is formatted according to the journal instructions, except where dissertation requirements conflicted.

In addition to the two included papers, this dissertation contains General Introduction and General Conclusions sections. Both sections were formatted according to instructions for the journal, Aquatic Toxicology. References cited in both sections follow Appendix D.

1. FACTORS AFFECTING CHOLINESTERASE ACTIVITY IN BLUEGILL (LEPOMIS MACROCHIRUS) BRAINS

A paper to be submitted to Aquatic Toxicology

Sheryl L. Beauvais, Gary J. Atchison, Michael Coffey

Abstract

Organophosphorus (OP) insecticides are extensively used to control crop pests, and can enter aquatic systems draining agricultural watersheds. Because OPs are highly toxic to aquatic organisms, monitoring is important, yet difficult due to the low persistence of OPs. Inhibition of activity in cholinesterase (ChE), a group of enzymes targeted by OPs, can be used to monitor exposure to OPs; however, high variability in ChE activity may interfere with the usefulness of this technique. Several potential sources of variation in ChE activity, including water temperature, collection method, and fish sex, were investigated using bluegill collected over two consecutive summers from backwater areas of the upper Mississippi River. No ChE inhibition was found in any of the samples, nor did ChE activity vary significantly by water temperature, collection method, or sex of fish.

Two methods of euthanasia, spinal cord severing and anesthesia with Finquel®, were compared in bluegill collected from a small lake, to determine potential effect on cholinesterase activity. The method of euthanasia did not affect ChE activity. Data from bluegill in the euthanasia experiment were pooled with data

from bluegill in the monitoring study to investigate potential effects of total length on ChE activity. Mean ChE activity varied inversely with fish total length.

Introduction

Organophosphorus insecticides (OPs) are extensively used throughout the world to control crop pests (Eto, 1991; Brunetto et al., 1992; Fairchild et al., 1992; Van Urk et al., 1993; ERS, 1994). OPs can enter aquatic systems that drain agricultural watersheds through routes such as direct application for aquatic insect pest control (Yasuno, 1991; Stevens and Warren, 1992), aerial overspray (Hennessey et al., 1992), and runoff from crop fields (Cooper, 1991; Richards and Baker, 1993; Dieter et al., 1995). Runoff from fields occurs with rain, and like aerial overspray, results in pulsed events with relatively short exposure periods.

Assessment of an aquatic system's exposure to OPs is difficult as these compounds are non-persistent due to rapid chemical and biological degradation (Weinberger et al., 1982; Wang et al., 1989; Brock et al., 1992). OPs tend to adsorb to sediments (Sharom et al., 1980; Knuth and Heinis, 1992), because of generally low water solubilities. For example, the solubility of chlorpyrifos is 1-2 mg/L at 25°C and its mean soil adsorption coefficient (K_d) is 173 mL/g (Racke, 1993). Therefore, OPs may be found in a river for only short intervals following rain events, adsorbed to particulates in runoff. Timing of sample collections and expense of analyses make direct monitoring of these insecticides problematic.

Because OPs are difficult and expensive to monitor in the field, a reliable indicator of exposure and resulting toxic effects is needed. Such an indicator must integrate across exposure events to provide evidence of prior exposure at sampling times. The indicator must also be sensitive to OP exposure at concentrations present during exposure events. Finally, it must be less expensive to measure than analyses of insecticide residues in water or animal tissue.

Monitoring of exposure is important because OPs are highly toxic to aquatic organisms (de Bruijn et al., 1991; Zinkl et al., 1991; Pavlov et al., 1992; Richmonds and Dutta, 1992). In bluegill (Lepomis macrochirus), for example, the 96-hr LC_{50} is 2.4 μg/L for chlorpyrifos and 1.8 μg/L for terbufos (Mayer and Ellersieck, 1986). Symptoms of acute poisoning in fish include muscle paralysis, hyperactivity and loss of equilibrium (Zinkl et al., 1991).

OPs cause acute toxicity primarily through cholinesterase (ChE) inhibition (Ware, 1994). Cholinesterase is a group of enzymes present in all animals, which hydrolyzes the neurotransmitter, acetylcholine, as well as similar compounds (Silver, 1974). OP binding to ChE is largely irreversible due to formation of a covalent bond (Matsumara, 1985), and new enzyme synthesis is often required for recovery from ChE inhibition. For this reason, ChE inhibition caused by OPs may persist for more than two to six weeks (van der Wel and Welling, 1989; Morgan et al., 1990; Carr et al., 1995). ChE activity inhibition in aquatic organisms has often been suggested as a way to demonstrate OP exposure (Weiss and Gakstatter,

1964; Coppage and Braidech, 1976; Habig and Di Giulio, 1991). Carbamate insecticides also inhibit ChE, but carbamate binding to ChE is reversible over relatively short intervals (Szeto et al., 1985); however, ChE inhibition can be useful for monitoring exposure to carbamates if assayed early enough following exposure (Coppage, 1977). Because carbamates are used in lesser quantities than OPs, the majority of monitoring is concerned with OPs.

Cholinesterase activity compares favorably with chemical analysis of insecticide residues as an indicator of OP exposure. ChE inhibition often persists for longer intervals than insecticide residues are detectable in water (Brock et al., 1992; Knuth and Heinis, 1992). ChE inhibition is a sensitive indicator of exposure to low OP concentrations: ChE activity is significantly reduced at OP concentrations as low as 1 μg/L. For example, rainbow trout (Oncorhynchus mykiss) exposed to 1 μg/L methidathion for 96 hr had 23% ChE inhibition (Flammarion et al., 1996). ChE analysis is less expensive than residue analysis and can be performed on crude tissue homogenates (Fairbrother et al., 1991), while residue analysis requires tissue extractions.

The major disadvantage of using ChE inhibition rather than residue analysis is that there is a high variability in ChE activity which confounds determination of whether enzyme activity is inhibited. There are differences between species, as well as different responses within a species to different ChE-inhibiting compounds (Habig and Di Giulio, 1991). Most serious, however, is the fact that ChE activity

differs among individuals of the same species. Causes of this variation are not fully understood.

Potential sources of variation include differences in age, sex, reproductive status, and environmental factors such as water temperature (Rattner and Fairbrother, 1991). ChE activity is often greater in younger, smaller organisms than in older, larger ones (Weiss, 1961; Rath and Misra, 1980; Zinkl et al., 1987; Gard and Hooper, 1993; Fishwick et al., 1996). Serum and whole body ChE activity has been shown to vary by sex and reproductive status in birds and fish, though the direction of variation differs among species (van der Wel and Welling, 1989; Rattner and Fairbrother, 1991); in contrast, no difference in brain ChE activity has been found by sex or reproductive status in birds or fish studied so far (Hogan, 1970; Coppage, 1972; Rattner and Fairbrother, 1991). In some studies, ChE activities have increased with increasing water temperature (Hazel, 1969; Hogan, 1970), while in others, water temperature did not appear to affect ChE activity (Zinkl et al., 1987; Cole, 1995). A potential source of variation which has not been investigated is use of an anesthetic for euthanasia: if the anesthetic inhibits ChE, then fish euthanized with the anesthetic may appear to have been exposed to an OP.

If the sources of individual variability in ChE activity can be understood, then such variability may be reduced in future monitoring programs through compensation for contributing factors. Factors which are not found to contribute to variation in ChE activity may be ignored, thus saving unnecessary efforts.

This study was done to investigate some of the major potential sources of variation, using a large data set from a monitoring project, as well as experiments involving locally collected bluegill (L. macrochirus). Bluegill collected alongside cornfields during two growing seasons as part of a monitoring project in the Mark Twain National Wildlife Refuge were analyzed for ChE inhibition. This data set was used to attempt to determine whether water temperature, fish size or fish sex affected brain ChE activity. Additional bluegill were collected from a small lake, and subjected to one of two methods of euthanasia, to determine effects on ChE activity. Results from this study will be used to provide recommendations for monitoring programs using ChE activity to monitor for OP exposure.

Methods

Monitoring for cholinesterase inhibitors in the upper Mississippi River

The Mississippi River is an important floodplain-river ecosystem that supports many aquatic and wetland biota (Fremling et al., 1989). Farming is a common activity along the upper Mississippi River (UMR), and contamination of the river by the most commonly used herbicides has been documented (Thurman et al., 1992). For monitoring potential contamination by ChE-inhibiting pesticides in the UMR, a logical choice is the bluegill sunfish (Lepomis macrochirus). Not only are there abundant bluegill populations throughout the Mississippi River (Fremling et al., 1989), but bluegill have often been used in laboratory and field studies of ChE

inhibitors (Weiss, 1961; Weiss and Gakstatter, 1964; Eaton, 1970; Macek et al., 1972; Vittozzi and De Angelis, 1991; Richmonds and Dutta, 1992; Tanner and Knuth, 1995), and have been found to be highly sensitive to OPs in comparison to other fish species (Mayer and Ellersieck, 1986; Vittozzi and De Angelis, 1991).

Adult bluegill from the UMR were collected from backwater areas of the Mark Twain National Wildlife Refuge along the Illinois shore (41° latitude, 91° longitude), about 677-688 km from the mouth of the Mississippi River. They were collected by personnel from the U. S. Fish and Wildlife Service or the Illinois Department of Conservation, using AC or DC-pulse electrofishing. One collection (2 May 1995) included trap netted fish and another collection (17 June 1995) consisted of fish captured by angling. These method changes were due to problems with high water, which made bluegill difficult to capture by electrofishing.

In 1995, all samples were collected from North Lake, River Kilometer 692. In 1996, samples were collected from Spring Slough, which is downriver of North Lake. Sampling in Spring Slough was done at both the upper end (River Kilometer 682) and the lower end (River Kilometer 681) of the slough on each sampling date, and water temperatures, pH and conductivity were taken at each location with a Solomat® water quality meter (Neotronics, Norwalk, CT).

At time of collection, the total length (mm) of each fish was measured and recorded. Fish were bagged individually in Ziploc® freezer bags, placed on ice immediately, then shipped on dry ice to the Department of Animal Ecology at Iowa

State University for ChE analysis. Fish were kept frozen until analysis, which generally occurred within 12 h of arrival at ISU in 1995; in 1996, fish were placed in a -80°C ultracold freezer until analysis, which occurred within 14 d. Numerous studies of long-term storage of fish and bird tissues and homogenates have demonstrated that storage at -20°C and below will maintain ChE activity over a period of several weeks (Fairbrother et al., 1991; Cole, 1995).

Variation in cholinesterase activity by water temperature

To determine whether water temperature influences ChE activity, water temperatures were taken when fish were collected in 1996 UMR sampling. During each of the four collection dates in 1996, samples were taken from both the upper and lower ends of Spring Slough; ChE means were calculated for samples collected at each of the eight temperatures.

Variation in cholinesterase activity by sex

Comparisons were made among adult bluegill from the UMR. Sex of bluegill was determined either by external characteristics for fish in breeding condition or by gonadal inspection.

Variation in cholinesterase activity by euthanasia method

To investigate potential effects of the use of an anesthetic, juvenile and adult bluegill were collected by seine or by AC electrofishing (on three separate occasions) from Horticulture Lake (HL). HL is a small impoundment (5 ha) at Iowa State University's Horticultural Research Station, Story County, Iowa (42°06' lat, 93°35' long). At the time of collection, water temperature and pH were recorded using a Horiba model U-10 water quality meter (Horiba Ltd., Kyoto, Japan).

Bluegill were divided into two groups; attempts were made to balance sizes between groups. Fish in one group were euthanized immediately by immersion for 15 min in 300 mg/L Finquel® (tricaine methanesulfonate; Argent Laboratories, Redmond, WA); fish in the other group were euthanized immediately by severing the spinal cord just behind the head with 10-cm stainless steel scissors. Fish were bagged individually in Ziploc® freezer bags and placed in a cooler of ice for transport to the laboratory. They were stored at -80°C until ChE analysis, which occurred within 6 weeks.

Variation in choiinesterase activity by fish total length

Comparisons were made between mean total length and mean ChE activity among juveniles from HL and adults from the UMR, across all dates. Fish were divided into size classes; each size class spanned 20 mm.

Cholinesterase analysis

ChE activities were assayed on individual, whole brains of L. macrochirus. Bluegill were removed from the -80°C freezer in small groups, and partially thawed in the refrigerator to soften brain tissue. Excess mucous was wiped from the exterior of the fish with paper towels and fish wet weight was determined to the nearest 0.1 g on a top-loading Ohaus Model CT-600S balance. Total length was recorded as written on freezer bags of adults from UMR, or measured just before dissection for juveniles from HL. Sex was determined for adults.

Each brain was removed with care, to include all lobes and exclude the optic nerves, and placed in 500 µL cold pH 7.4 Tris buffer (all reagents were purchased from Sigma, St. Louis, MO). Brains were weighed to the nearest 0.1 mg on a Sartorius A200S analytical balance, poured into a glass homogenizer tube (Glas-Col, Terre Haute, IN) along with a proportional volume of pH 7.4 Tris buffer to give a 100-fold dilution, and then homogenized with Teflon pestle (Glas-Col, Terre Haute, IN) driven by a stirring motor.

During the first analyses of ChE activity in brains of juveniles, which occurred when the relative humidity was low, enough of the initial 500 µL cold pH 7.4 Tris buffer evaporated between the first weighing (weigh boat plus buffer, before fish dissection) to seriously affect the apparent brain weight. In as little as 10 min, the weight of Tris buffer alone could change by as much as 0.002 g. During times of high relative humidity, condensation of atmospheric moisture into the cold buffer

could change buffer weight just as quickly. This was not a serious error with adult brains, but with the much smaller juvenile brains the error was sufficient to invalidate the measurement. In all subsequent assays, control weigh boats containing only Tris buffer were carried through both weighings along with sample weigh boats. Samples were discarded if the change in weight of the control weigh boats was greater than 10% of the apparent brain weight.

The homogenate was stored on ice or refrigerated until analysis. Total ChE activity was measured; no differentiation between acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities was made. ChE analysis was done with a colorimetric method, modified to use a THERMOmax microplate reader and SOFTmax software (Molecular Devices Corp., Menlo Park, CA) to monitor the rate of formation of a yellow product, 5-thio-2-nitrobenzoate, from a reaction of 5,5-dithiobis-2-nitrobenzoic acid with thiocholine released by cleavage of acetylthiocholine by ChE (Ellman et al. 1961; Hill and Fleming, 1982; Gard and Hooper, 1993). Increase in absorbance at 405 nm was monitored for 2 min at 25°C with readings every 8 s. The software calculated each maximum reaction rate (Vmax) by doing a series of regressions on the 16 data points. ChE activities were calculated from Vmax and dilution factors, and reported as micromoles acetylthiocholine hydrolyzed per min per g of brain tissue (μM AThCh hydrolyzed/min/g brain tissue). Reactions were optimized at 25°C (maintained by an incubator in the microplate reader) and 3.16×10^{-3} M acetylthiocholine.

Homogenates were analyzed in triplicate. Each homogenate was vortexed for 10 s immediately prior to pipetting triplicates into a microplate. Analysis of a homogenate was repeated if the triplicate coefficient of variation (CV) was greater than 10%. With each analytical run, a triplicate of a check standard made from pooled homogenates, and stored in cryovials in liquid nitrogen, was also analyzed. A run was repeated if the check standard CV was greater than 10%. See Appendix A for a Standard Operating Procedure and Appendix B for results of check standard assays.

Statistics

Normal distribution of ChE activities within each comparison was checked using the Shapiro-Wilk statistic, W, in PROC Univariate (SAS, 1993). T-tests were done to compare ChE activities of bluegill collected by trap net and angling with bluegill collected on the same or adjacent days by electrofishing. Comparisons among means were made using univariate analysis of variance (ANOVA) and linear regressions in PROC REG as appropriate. Univariate ANOVA was used to test for differences between males and females, and for differences in methods of euthanasia. Contrast results were considered significant at an α level of 0.05.

Potential effects of collection date and size were difficult to separate in fish collected from the UMR and HL, as each sample contained fish of differing sizes. Between collection dates, not only did the water temperature change, but fish age

and reproductive state also differed. For this reason, fish from each sample collection were divided into size classes by total length. A linear regression was done of mean ChE activity vs. mean total length using PROC REG (SAS, 1993).

Results

Monitoring for cholinesterase inhibitors in the upper Mississippi River

The sampling periods covered May 1-June 30, 1995, and April 23-August 12, 1996. Water temperatures at the UMR sampling site varied from 15.8-25.5°C in 1995, and from 13.0-25.9°C in 1996. Dates in which precipitation greater than 2 cm was recorded are listed in Table 1.1. Every sample collection date, other than the last two in 1996, was preceded by a rain event of at least 2 cm in the previous 2 weeks. Contamination of the study site was inferred from the proximity of fields which were being treated with insecticides and the occurrence of rain events. Information regarding identity and application dates of insecticides used in adjacent fields could not be determined, due to the reluctance of applicators to provide detailed information.

Sampling was done on nine different days in 1995 and four different days 1996. A total of 221 adult bluegill were collected, 131 in 1995 and 90 in 1996 (Table 1.2). There was no evidence of ChE inhibition in the thirteen means by collection date; mean activities (8.25-12.85 μM substrate hydrolyzed/min/g brain tissue) were similar to a literature value for uninhibited bluegill ChE (Richmonds and

Table 1.1: Water quality data from sampling sites in the upper Mississippi River. Water quality was coordinated with fish sampling in 1996, but not in 1995. Rainfall data includes dates on which more than 2 cm was recorded.

Collection Time	pH	Water Temp (°C)	Conductivity (μS)
1995: (North Lake)			
April 19, 3:30 PM	8.5	15.8	297
May 15, 9:30 AM	8.4	17.7	354
June 6, 1:57 PM	8.4	25.0	286
June 27, noon	7.9	25.5	302
1996: (Upper, Lower Spring Slough)			
April 23	9.1, 8.9	16.2, 13.0	392, 362
May 21	7.9, 8.1	20.5, 19.5	352, 352
July 17	8.0, 8.1	25.1, 24.5	366, 375
August 12	8.8, 8.1	25.9, 23.5	328, 353

Rain Events[a] Date	Amount (cm)
1995:	
April 11	2.69
May 8	4.70
May 23-24	6.22
June 2	3.12
June 27	3.18
1996:	
April 15	2.46
April 29	3.56
May 9-10	16.15
May 17	3.23
May 27-28	5.66
July 21	2.77

[a] Rain event data provided by Dr. Steven Hollinger of the Illinois Water Survey, from a station at Keithsburg, IL, about 5 km downstream of sample site.

Table 1.2. Comparison of mean total length and mean brain cholinesterase activity between collection dates among adult bluegill collected from the upper Mississippi River. Standard deviations are given in parenthesis behind each mean.

Collection Date	N	Mean Total Length (mm)	Mean Brain ChE Activity [a]
1995:	131	161 (17.0)	9.73 (2.560)
May 1	11	163 (14.0)	8.58 (2.732)
May 2	44	163 (19.0)	8.25 (1.918)
May 19	11	169 (14.7)	10.2 (1.659)
June 5	13	163 (19.3)	10.6 (2.407)
June 15	16	155 (10.2)	11.8 (3.052)
June 16	9	147 (24.3)	11.0 (1.753)
June 17	7	154 (15.7)	8.66 (1.855)
June 27	10	160 (15.0)	10.7 (2.296)
June 30	10	167 (10.6)	11.1 (2.192)
1996:	90	147 (20.0)	11.38 (2.350)
April 23	22	144 (19.9)	10.90 (2.749)
May 21	17	133 (22.3)	9.27 (1.665)
July 17	24	152 (15.9)	11.67 (1.415)
August 12	27	152 (16.9)	12.85 (1.971)

[a] Cholinesterase activities are reported as μM AThCh hydrolyzed/min/g brain tissue

Dutta, 1992). The overall mean ChE activity for both years was 10.40 ± 2.60 μM substrate hydrolyzed/min/g brain tissue. The overall mean total length was 155 ± 20 mm.

Because bluegill in two samples were collected by different methods, t-tests were done to determine whether the mean ChE activities differed for these samples. The mean ChE activity of bluegill collected on 2 May 1995 by trap net (7.98 μM substrate hydrolyzed/min/g brain tissue) did not differ significantly (P=0.22) from the mean ChE activity of bluegill collected the same day by electrofishing (8.72 μM substrate hydrolyzed/min/g brain tissue). The mean ChE activity of bluegill collected on 17 June 1995 by angling (8.66 μM substrate hydrolyzed/min/g brain tissue), however, was significantly lower (P=0.01) than the mean of bluegill collected on 15 and 16 June 1995 by electrofishing (11.55 μM substrate hydrolyzed/min/g brain tissue). Based on these results, data from bluegill collected by trap net were included in comparisons to evaluate changes in ChE activity by sampling date, while data from bluegill collected by angling were not included.

Bluegill collected in 1996 tended to be smaller and to have higher ChE activities (Table 1.2) than bluegill from 1995 samples. In both years, mean ChE activities tended to increase across sampling dates; when mean ChE activities were plotted against the Julian date (Figure 1.1), the gradual increase was apparent. A regression of these data pooled across both years gave the following equation: (mean ChE) = 6.19 + 0.027(date). This regression was significant (P=0.0018), with

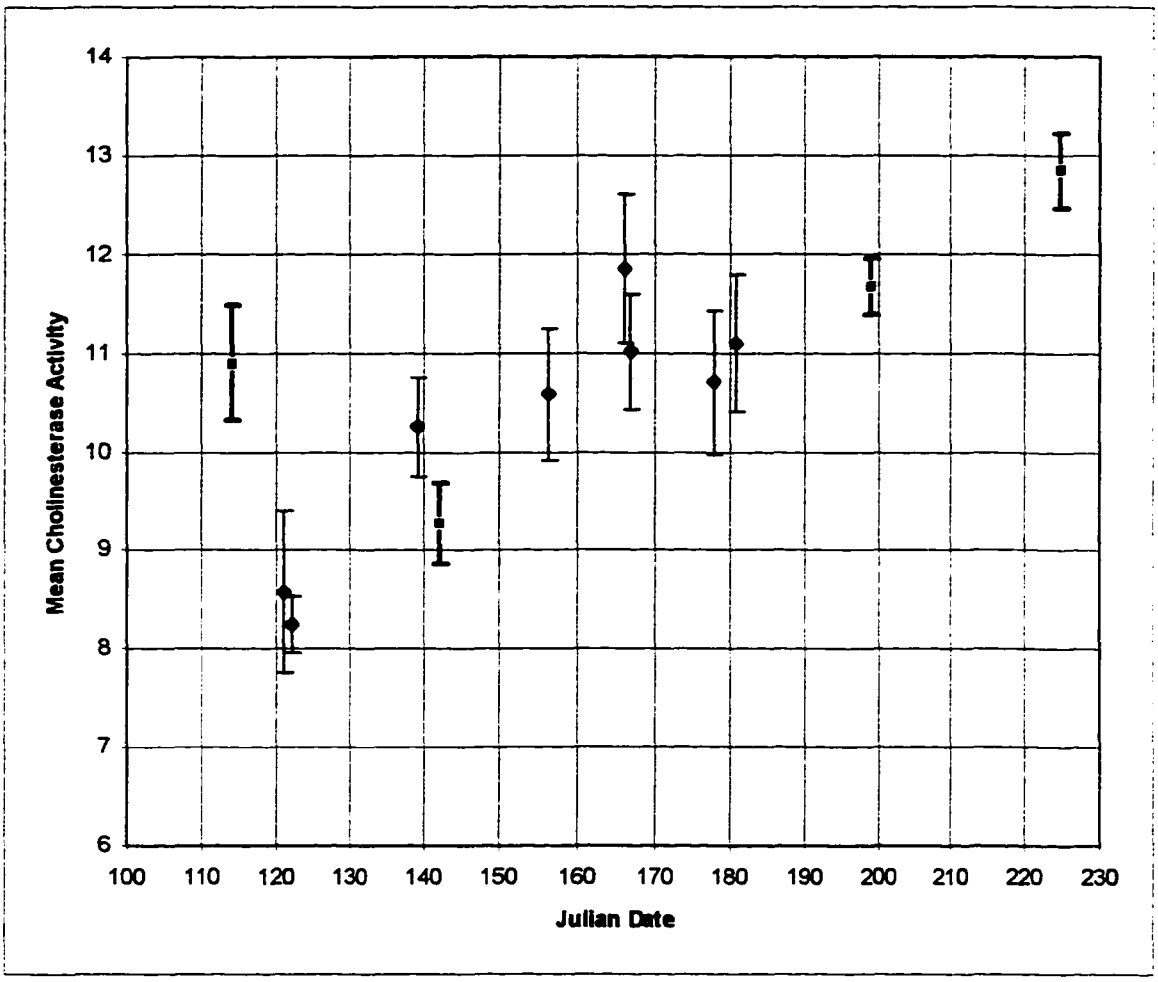

Figure 1.1. Mean (± SE) cholinesterase activity (μM substrate hydrolyzed/min/g brain tissue) of adult bluegill collected from the upper Mississippi River in 1995 (diamonds) and 1996 (squares). Means are organized by Julian date: day 114 corresponds to April 23, and day 225 corresponds to August 12.

$r^2 = 0.64$. A linear regression of the 1995 collections also gave a significant equation (P=0.004), with a better fit than the equation for data pooled across both years ($r^2 = 0.77$). The equation for the regression of 1995 data was, (mean ChE) = 3.21 + 0.046(date). A regression of the 1996 data alone was not significant (P=0.19), though the fit of the equation ($r^2 = 0.65$) was as good as for the equation of data from both years; this was probably due to the fact that only four collections were done in 1996.

Variation in cholinesterase activity by water temperature

Comparisons of ChE activity and water temperature in adults from UMR were made among eight means of samples collected in 1996 (Figure 1.2). There were eight means on four sampling days because bluegill were collected from two sites; temperatures differed by 0.5-3.2°C between sites on a sampling day. Water temperatures at the two sites ranged from 13.0°C to 25.9°C across all days. The mean ChE of the eight samples was 11.16 $\pm$ 1.29 μM substrate hydrolyzed/min/g brain tissue. A regression of mean ChE activity on water temperature was not significant (P=0.19). There was no discernible effect of temperature on ChE activity.

Variation in cholinesterase activity by sex

A univariate ANOVA compared mean ChE activity between males and females among bluegill adults from the UMR by each collection date (Table 1.3).

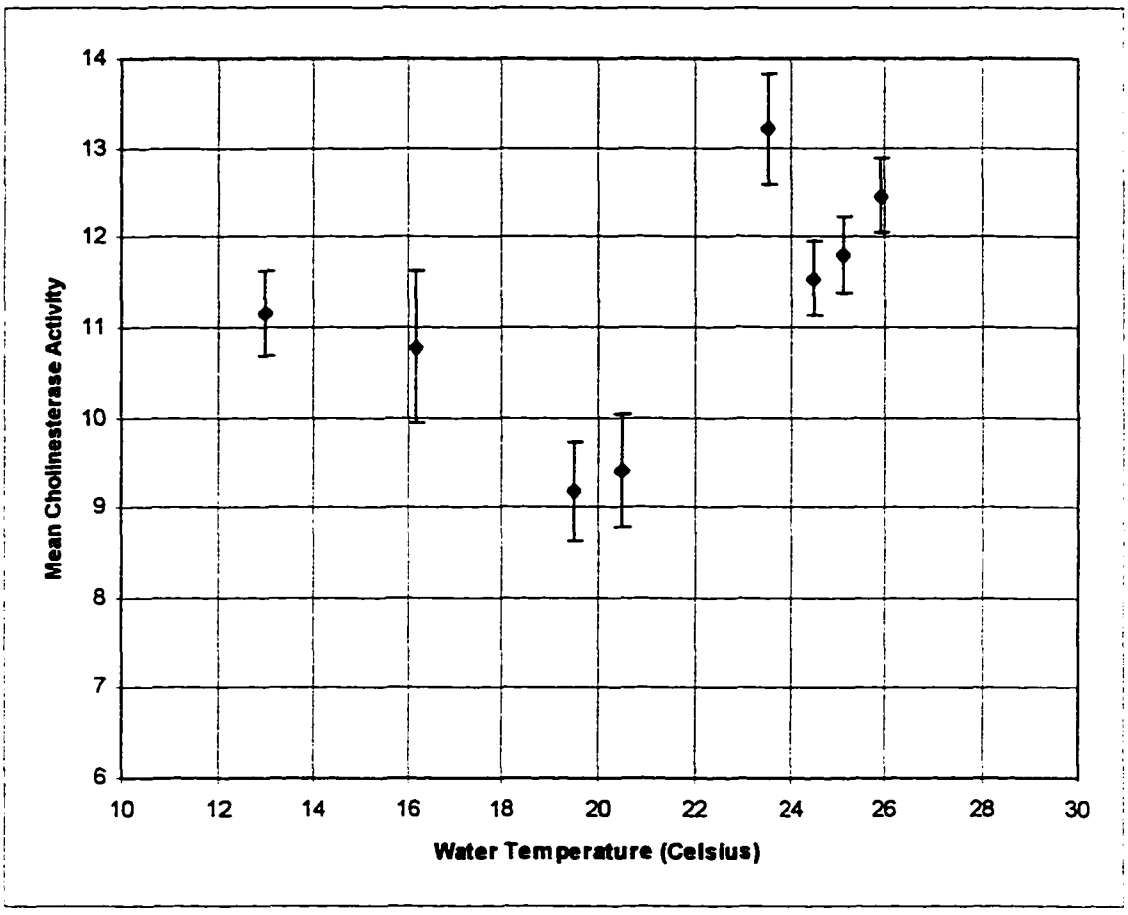

Figure 1.2. Mean (± SE) cholinesterase activity (μM substrate hydrolyzed/min/g brain tissue) of bluegill collected at different water temperatures from the upper Mississippi River in 1996.

Table 1.3. Mean brain cholinesterase activities by collection date for male and female bluegill collected from the upper Mississippi River in 1995 and 1996.

Date	Number of Males	Mean ChE[a]	SEM of ChE	Number of Females	Mean ChE[a]	SEM of ChE
1995:						
May 2	13	9.14	0.429	10	8.00	0.615
May 19	6	10.42	0.852	5	10.06	0.341
June 5	8	10.22	0.995	5	11.17	0.748
June 15	5	12.60	0.947	11	11.51	1.007
June 16	8	11.19	0.632	1	9.60	0
June 27	8	10.04	0.640	2	13.36	1.414
June 30	6	10.80	0.743	4	11.52	1.456
1996:						
April 23	5	11.46	1.638	17	10.74	0.615
May 21	11	9.61	0.540	6	8.66	0.541
July 17	14	10.01	0.326	10	11.19	0.503
August 12	18	12.98	0.497	9	12.59	0.582

[a] Cholinesterase activities are reported as μM AThCh hydrolyzed/min/g brain tissue

Samples from 15 June 1995 were excluded from the comparison because only one female was collected. There were a total of 83 females and 106 males in the comparison. Mean ChE activities were 10.60 ± 2.57 μM substrate hydrolyzed/min/g brain tissue for females and 10.96 ± 2.35 μM substrate hydrolyzed/min/g brain tissue for males. There was no significant difference in mean ChE activity between males and females (P=0.95).

Variation in cholinesterase activity by euthanasia method

Comparisons were made in mean ChE activity between bluegill euthanized by severing of the spinal cord and by anesthesia with 300 mg/L Finquel®. Mean ChE activity for fish euthanized by spinal cord severing was 17.81 ± 8.19 μM substrate hydrolyzed/min/g brain tissue; mean ChE activity for fish euthanized by Finquel® was 18.11 ± 7.60 μM substrate hydrolyzed/min/g brain tissue. There was no significant difference in mean ChE activity between by method of euthanasia (P=0.80).

Variation in cholinesterase activity by fish total length

During each sampling of fish from the UMR, attempts were made to collect only fish having total lengths 150-170 mm. High water conditions in both years increased the difficulty of collecting bluegill; the range of bluegill collected was actually 95-197 mm. To provide an estimate of variation in ChE activity by total

length in bluegill, fish collected from the UMR were divided into four size classes by total length: 110-129, 130-149, 150-169 and 170-189 mm (Figure 1.3). Seven fish were outside these ranges. Bluegill collected from HL were also divided into size classes; four fish were 110-129 mm, five were 130-149 mm, and none were larger. Three classes were added to accommodate juveniles and small adults from HL: 30-49, 70-89 and 90-109 mm. Overall means of each of the seven size classes are displayed in Figure 1.4. A linear regression of mean ChE activity on mean total length across 41 means, yielded a significant regression (P=0.0001) and a moderate fit (r^2=0.44) for the equation, (mean ChE) = 18.33 - 0.049 (mean Total Length). Additional statistical tests showed that a linear regression was an adequate model, and that a single line was adequate to fit ChE activity in fish from both HL and UMR. In general, mean ChE activity varied inversely with fish total length.

Discussion

Monitoring for cholinesterase inhibitors in the upper Mississippi River

There was no evidence of ChE inhibition in any of the samples collected in either of the two years. In this study, water samples were not analyzed for pesticide contamination; the potential for contamination was inferred from the proximity of fields being treated with insecticides. No information was available about when or what insecticides were applied to these fields. It is possible that no contamination by ChE inhibitors occurred during this study.

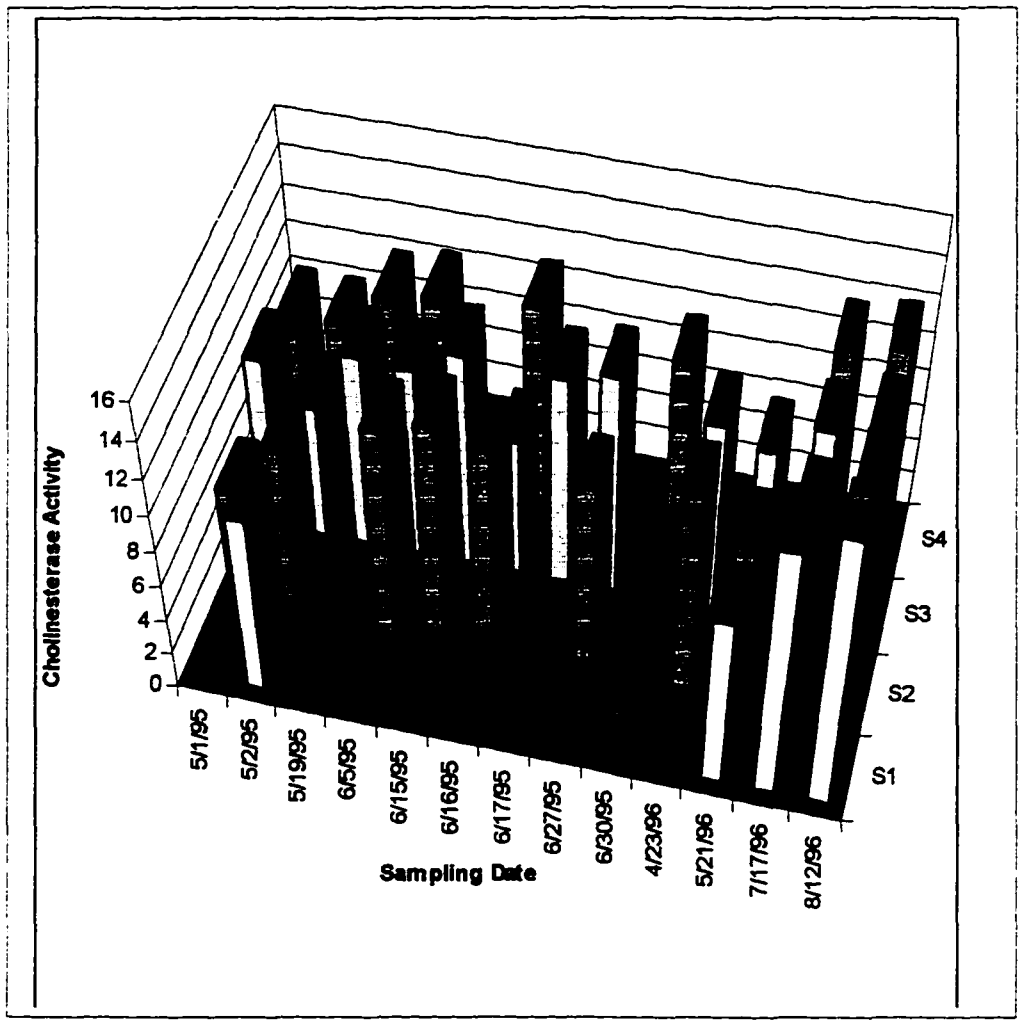

Figure 1.3. Mean brain cholinesterase activity in adult bluegill sampled from the upper Mississippi River, organized by collection date. Note that the first nine dates were in summer, 1995, while the last four dates were in 1996. Fish sampled on each date have been split into four size classes: S1 = 110-129 mm; S2 = 130-149 mm; S3 = 150-169 mm; S4 = 170-189 mm. Where cholinesterase activity appears to equal zero, no fish of that size class were collected.

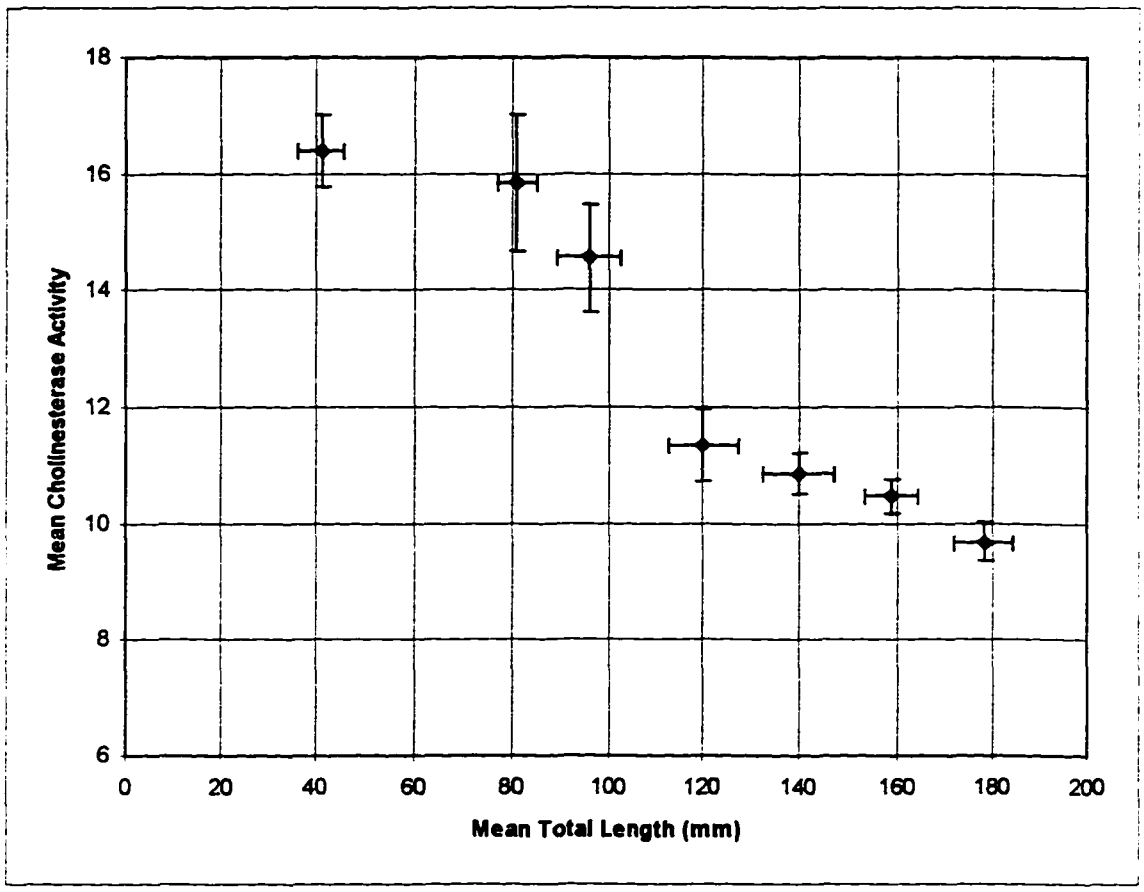

Figure 1.4. Mean (± SE) brain cholinesterase activity by size (total length) class in adult and juvenile bluegill. The three smallest size classes (30-49 mm; 70-89 mm; 90-109 mm) contain bluegill collected from Horticulture Lake, the two largest size classes (150-169 mm; 170-189 mm) contain bluegill collected from the upper Mississippi River, and the two middle size classes (110-129 mm; 130-149 mm) contain bluegill from both places.

In earlier studies where ChE inhibition was detected in fish collected from waters either presumed or known to be contaminated with ChE inhibitors, the concentration of ChE inhibitors was probably quite high. Analysis of brain ChE activity in fish taken from bodies of water contaminated with ChE inhibitors detected inhibition where the contamination was due to effluent discharged by manufacturers of organophosphorus and carbamate insecticides (Williams and Sova, 1966; Coppage and Braidech, 1976), or was from spraying of OPs into shallow ponds (Thirugnanam and Forgash, 1977; Lockhart et al., 1985). If ChE inhibitors did contaminate the monitored backwater areas, there may have been sufficient dilution along with rapid degradation to prevent occurrence of ChE inhibition in bluegill.

The possibility exists that bluegill may have been exposed to OPs, but recovered ChE activity prior to sampling. This would be more likely in 1996, when collections were a few to several weeks apart. In 1995, however, sampling occurred approximately every two weeks. In previous studies documenting ChE inhibition by OPs, significant inhibition was still seen after two weeks (van der Wel and Welling, 1989; Carr et al., 1995). Inhibition should have been detected in 1995, if it occurred.

Variation in cholinesterase activity by water temperature

In the present study, water temperature did not significantly affect brain ChE activity. This may be due to the fact that water temperatures were measured only

when fish were collected, and would fluctuate over time and space; the temperatures to which fish were acclimated may have been lower, especially in spring when temperatures increase rapidly. Also, fish may have been in microclimates where water temperatures were lower than recorded near the surface.

Studies in which ChE activity was found to vary with water temperature involved greater temperature spans than in the present study; also, their lowest temperatures were 5°C or lower. Hazel (1969) determined that brain ChE activity was more than twice as high for goldfish (Carassius auratus) at 35°C compared to 5°C; ChE activity declined above 35°C. In bluegill, Hogan (1970) found significant regressions between mean air or water temperatures and brain ChE activity, but the bluegill in that study were acclimated to seasonal water temperatures (0-27°C) and sampled throughout the year. Variation seen by Hogan (1970) may also have been due to seasonal factors other than temperature, such as reproductive condition. Baldwin and Hochachka (1970) determined that in rainbow trout (Onchorhynchus mykiss), brain ChE occurred in two variants, a "cold" variant in trout acclimated to 2°C, and a "warm" variant in trout acclimated to 17°C. Both variants were seen in trout acclimated to an intermediate temperature.

Differences in ChE activity were not seen in studies involving warmer temperatures and temperature ranges of less than 12°C. Rainbow trout exposed for seven days to temperatures of 9, 13, or 20°C had similar brain ChE activities to one another (Zinkl et al., 1987). Similarly, Cole (1995), found no significant differences

in bluegill ChE activity by temperature in bluegill acclimated to five temperatures between 20 and 30°C for a minimum of two weeks. Temperature ranges in these latter two studies were similar to the temperatures in the present study (13.0-25.9°C), and may simply be insufficient to manifest changes in ChE activity. However, in a monitoring program for OPs that would take place in the spring and summer, a temperature span of 15-30°C is reasonable for bluegill and seemingly would not affect ChE activity.

Variation in cholinesterase activity by sex

There was no significant difference in brain ChE activity found between male and female adults collected from the UMR. This result is in agreement with Hogan (1970), who also found no significant difference in brain ChE activity by sex in bluegill. Coppage (1972) found no significant difference in brain ChE activity by sex in sheepshead minnows (Cyprinidon variegatus).

In guppies (Poecilia reticulata) exposed to chlorpyrifos, both unexposed and exposed males had significantly higher whole body ChE activities than corresponding females (van der Wel and Welling, 1989). However, whole bodies of guppies were analyzed; females may have had a lower proportion of their body weight as tissue containing ChE. In the present study, as in the other studies just cited (Hogan, 1970; Coppage, 1972), brain tissue was analyzed.

Based on these results, it does not appear to be necessary to segregate samples by sex in bluegill for monitoring studies using brain ChE activity.

Variation in cholinesterase activity by euthanasia method

There was no significant difference in brain ChE activity found between bluegill euthanized by spinal cord severing and bluegill euthanized with Finquel®. This is apparently the first time this factor has been investigated in vivo, in spite of the fact that use of anesthetics is common. The chemical investigated in the present study, or Finquel® (formerly known as MS-222), is a common anesthetic used on fish. The mode of action of Finquel® is unknown; however, it is likely that it inhibits neurotransmitter release (Yuwiler and Samuel, 1974). When an anesthetic is used in a study, generally all fish in the study are treated with the anesthetic (Eaton, 1970; Finlayson and Rudnicki, 1985). Therefore, if the anesthetic has any effect on ChE activity, it may not be detected; if that were the case, comparison between studies using different euthanasia methods could be invalidated.

The effect of Finquel® on ChE activity has been investigated in an in vitro study, where muscle from fathead minnows (Pimephales promelas) was incubated with Finquel®. At Finquel® concentration of 0.013 M, or 3.4 g/L, ChE activity was 50% inhibited (Olson and Christensen, 1980). That concentration required to inhibit ChE activity by 50% was more than ten times the concentration used for euthanasia

in the present study. The concentration of Finquel® required for euthanasia is insufficient to interfere with results of ChE assays.

Variation in cholinesterase activity by fish total length

Mean ChE activity varied inversely with fish total length. Total length is a sensible measure of size to use in comparisons. Generally, wet weight is used in size comparisons (Rath and Misra, 1980; Zinkl et al., 1987). However, adults' wet weight is strongly influenced by their reproductive status; adults collected from the UMR were in various reproductive conditions.

Brain ChE activity has been found to vary inversely by fish size in other studies. Brain ChE activity was inversely proportional to brain weight in several species of fish, including bluegill (Weiss, 1961). Most of the variation occurred in the smallest fish; adult fish did not vary much in brain ChE activity with brain weight (Weiss, 1961). In tilapia (Tilapia mossambica), a strongly negative, linear correlation was found between body weight and both brain and liver ChE activity (Rath and Misra, 1980).

The difference in ChE activity by size may, at least in some species, be a difference by age. Small rainbow trout (O. mykiss), with mean weights of 5, 15 and 54 g, had significantly lower brain ChE activities than large trout of 1300 and 2300 g (Zinkl et al., 1987). The difference was not linear, however; there was no significant difference in brain ChE activities between the three groups of small trout, nor was

there a significant difference between the two groups of large trout (Zinkl et al., 1987). In birds, brain ChE activity increases as fledglings mature (Grue and Hunter, 1984; Gard and Hooper, 1993). In wild mice and field voles, brain ChE increases during the first few weeks of life, then decreases until adulthood (Fishwick et al., 1996).

In the present study, juvenile bluegill apparently have much higher ChE activities than adults (Figure 1.4). However, it was not possible to test the relationship statistically, as no determination was made of fish age, and sexual maturity was not determined in fish in the intermediate size classes (70-89 and 90-109 mm). Graphs of brain ChE activity plotted against brain weight in Weiss (1961) show an apparent difference between adults and juveniles that agrees with results in the present study. Because most adults came from UMR, while juveniles came from HL, the possibility of differences in ChE activities between the populations cannot be ruled out with these data, though the fact that the same linear regression describes data from both places does not support a population difference.

Because of the effect of size on ChE activity, fish used to monitor exposure to ChE inhibitors should be as similarly sized as possible. In bluegill in this study, mean ChE activities differed significantly between size classes spanning only 20 mm. Difficulties in fish collection may frustrate attempts to limit this source of variation, as occurred in the present study. In such cases, relationships between size and ChE activities should be well enough characterized for the population that

estimates can be made of variation in ChE due to variation of size within fish sampled.

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2. USE OF CHOLINESTERASE ACTIVITY TO MONITOR EXPOSURE OF *CHIRONOMUS RIPARIUS* (DIPTERA: CHIRONOMIDAE) TO A PESTICIDE MIXTURE IN HYPOXIC WETLAND MESOCOSMS

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Abstract

Larvae of *Chironomus riparius* were exposed to hypoxia (dissolved oxygen concentrations of 0.5 - 4 mg L^{-1}) in wetland mesocosms and in a laboratory experiment. Exposure to hypoxia for up to 35 hours did not alter cholinesterase activity.

C. riparius larvae were exposed to concurrent dosing of the organophosphorus insecticide, chlorpyrifos, and the herbicides, atrazine and metolachlor, in both hypoxic wetland mesocosms and under normoxic conditions in a laboratory experiment. Exposure to atrazine or metolachlor did not cause ChE inhibition. There was no significant difference in ChE inhibition between larvae exposed to a mixture of chlorpyrifos, atrazine and metolachlor and larvae exposed to chlorpyrifos alone.

Chironomids appear to be good candidates for monitoring of cholinesterase inhibitors in shallow aquatic systems. Cholinesterase activities were significantly depressed following all doses of chlorpyrifos; chironomid cholinesterase activities are a sensitive indicator of exposure to cholinesterase inhibitors.

Introduction

Wetlands, transitional zones between upland and open water areas (Catallo, 1993), have both economic and ecological importance due to their high productivity and capacity to filter and store water (Murkin, 1989; Hook, 1993). Such areas are often exposed to mixtures of pesticides (ERS, 1994), yet toxicity tests are generally done as individual chemical exposures (Thompson, 1996). Organophosphorus insecticides (OPs) and herbicides such as atrazine are among the pesticides which contaminate wetlands (Neely and Baker, 1989).

OPs are extensively used throughout the world to control crop pests (Eto, 1991; ERS, 1994). OPs can enter aquatic systems that drain agricultural watersheds through routes such as direct application for aquatic insect pest control (Yasuno, 1991; Stevens & Warren, 1992), aerial overspray (Hennessey *et al.*, 1992), and runoff from crop fields (Cooper, 1991; Richards & Baker, 1993; Dieter *et al.*, 1995). Because insecticides that target aquatic pests are formulated for greater effectiveness in water, direct application can result in higher concentrations (e.g., up to 10 $\mu g\ L^{-1}$; Yasuno, 1991), and relatively longer-term exposures (e.g., 2-6 weeks; Yasuno, 1991) than those resulting from other uses. Runoff from fields occurs with rain, and results in pulsed events with relatively short exposure periods.

OPs are highly toxic to aquatic insects, including insects common in wetlands (Fairchild *et al.*, 1992; Fisher *et al.*, 1993; van Wijngaarden *et al.*, 1996). For example, the 24-hr LC_{50} of chlorpyrifos was 3.7 $\mu g\ L^{-1}$ to *Chironomus tentans* (Karnak & Collins, 1974), and the 24-hr LC_{50} of parathion and malathion to *C.*

riparius were 2.5 and 1.9 μg L^{-1}, respectively (Estenik & Collins, 1979). OPs sprayed into experimental wetlands in late May significantly decreased populations of aquatic invertebrates; predatory insect populations were most severely depleted, and did not recover that summer (Yasuno, 1991).

OPs cause acute toxicity primarily through cholinesterase (ChE) inhibition (Matsumara, 1985). Cholinesterase is a group of enzymes present in all animals, which hydrolyzes the neurotransmitter, acetylcholine, as well as similar compounds (Silver, 1974). OP binding to ChE is largely irreversible due to formation of a covalent bond (Matsumara, 1985), and new enzyme synthesis is often required for recovery from ChE inhibition. For this reason, ChE inhibition caused by OPs may persist for more than two to six weeks (Morgan *et al.*, 1990; Carr *et al.*, 1995). ChE inhibition in aquatic organisms has been suggested as a way to demonstrate OP exposure (Day & Scott, 1990; Varela & Augspurger, 1996).

ChE inhibition often persists for longer intervals than insecticide residues are detectable in water (Brock *et al.*, 1992; Knuth & Heinis, 1992). ChE inhibition is a sensitive exposure indicator of low OP concentrations; for example, exposure for 24 h to 1 μg L^{-1} fenitrothion caused 40% ChE inhibition in the amphipod, *Gammarus pulex* (Kuhn & Streit, 1994).

Agricultural watersheds typically contain more than one type of crop, each requiring different pesticides (ERS, 1994). For this reason, exposure of aquatic insects to OPs may also occur along with exposure to other pesticides. Each pesticide may be toxic to aquatic insects; toxicity of mixtures may be simply additive,

or less than or greater than additive. The top two pesticides applied to field crops in the United States in 1993 were the herbicides, atrazine and metolachlor (ERS, 1994). Atrazine has a low toxicity to aquatic invertebrates; its 48-h LC_{50} in first-instar *C. riparius* is 720 μg L^{-1} (Macek *et al.*, 1976), while in second-instar the 10-d LC_{50} was found to be above the solubility of the compound at 30 mg L^{-1} (Taylor *et al.*, 1991). No information is available in the literature on the toxicity of metolachlor to aquatic insects.

While few toxicity tests have been done on pesticide mixtures, there is a report of synergism between atrazine and an OP. A mixture of 10 mg L^{-1} atrazine and 15 μg L^{-1} parathion caused 73% mortality to larvae of the mosquito, *Aedes aegypti*; 10 mg L^{-1} atrazine alone caused no mortality, while 15 μg L^{-1} parathion caused 20% mortality (Liang & Lichtenstein, 1974). No mechanism for the increased toxicity was suggested by Liang & Lichtenstein (1974).

In addition to exposure to pesticide mixtures, wetland insects also face stress from low dissolved oxygen. Wetlands are often hypoxic because of shading by floating and emergent vegetation and high oxygen demand from decomposing plant litter (Buscemi, 1958; Rose & Crumpton, 1996). Effects of hypoxia on ChE activity have not been studied in aquatic insects. However, one experiment with trout (*Oncorhynchus mykiss*) exposed to the OP, dichlorvos, resulted in increased ChE inhibition under hypoxic conditions (Hoy *et al.*, 1991).

ChE activity in wetland insects can be used to monitor exposure of wetlands to OPs; however, it is important to know whether ChE activities are affected by

concurrent exposure to other common pesticides, as well as by hypoxia. To investigate these questions, larval chironomids (*C. riparius*) were exposed to hypoxia in a laboratory experiment, and an experiment was done with caged larval chironomids in wetland mesocosms. Chironomids are a good choice for monitoring in wetlands because larvae are easily reared in the laboratory, they tolerate hypoxia fairly well, and can be placed in wetlands in small cages, from which they can be retrieved at various times and tested for ChE activity.

The goal of this study was to ascertain if ChE activity in chironomids can be used as a sensitive biomarker to detect influx of OP insecticides into wetland mesocosms. Three objectives were set to meet this goal: determine whether chironomid ChE activity was affected by exposure to hypoxia, determine whether ChE inhibition by chlorpyrifos was increased with concurrent exposure to the herbicides, atrazine and metolachlor, and ascertain if caged chironomid larvae in wetland mesocosms had inhibited ChE activity in response to spraying of a pesticide mixture into the mesocosms.

Materials and methods

Test animals

C. riparius larvae in all experiments came from in-house cultures. Cultures were initiated in 1993 with eggs from the Midwest Science Center in Columbia, Missouri, and were maintained as continuous (i.e., neither eggs nor adults were

routinely removed to new cultures), static cultures in 40-L aquaria. See Appendix C for chironomid culture standard operating procedures.

Larvae used in experiments were second- to fourth-instar, with wet weights of 0.7-6.2 mg (mean = 2.4 $\pm$ 1.2; n = 255) in mescosm experiments and 1.0-5.9 mg (mean = 3.2 $\pm$ 0.9; n = 186) in laboratory experiments.

Wetland mesocosms

Wetland mesocosms were located at Hinds Research Center in Story County, Iowa. Mesocosms consisted of 3.35 m diameter, 0.9 m deep, polyethylene tanks sunk into the ground; tank rims were less than 0.5 m above ground. Construction of mesocosms occurred in 1989 (Rose, 1996), and was described in Crumpton *et al.* (1993). Tanks were individually valved, and around the upper rim of each mesocosm was a perforated polyvinyl chloride ring; water and solutions were sprayed into mesocosms through these rings.

Mesocosms contained mature stands of *Typha* (60-80 stems m^{-2}; Rose, 1996), except for three in which *Typha* was removed for the mixed pesticide experiment (see below). Mesocosm substrates consisted of wetland sediment, detritus and *Typha* roots, (root masses were removed in mesocosms cleared of *Typha*). Mesocosms were filled with well water to give water levels several cm above the substrate on the day before each experiment was started. Water within the mesocosms was circumneutral (pH range 6.8-7.1), with conductivity between 0.75 and 0.98 mS.

C. riparius larvae were placed in small cages, which were submersed in the mesocosms. Cages consisted of 12-mL plastic vials with three 1-cm holes (one in the top and two in sides), screened with 1.7 mm Fiberglas mesh, and covered with cotton cloth to prevent chironomid escape through the mesh. After exposure in the mesocosms, chironomids were removed and transported to the laboratory in water siphoned from a mesocosm not used in the experiment, to maintain hypoxic conditions until ChE analysis.

Effect of hypoxia on cholinesterase activity

Two experiments were run to determine whether exposure to hypoxia for up to 24 h can affect ChE activity. The first was a laboratory experiment to determine whether duration of hypoxia influenced ChE activity. Four *C. riparius* larvae were placed in each BOD bottle, along with carbon-filtered water through which nitrogen gas had been bubbled for 1 h. Each replicate consisted of three BOD bottles; one replicate was kept closed for each interval of 2, 4 and 6 h. Controls consisted of four larvae in each of three BOD bottles containing carbon-filtered water that had not been treated with nitrogen gas. Dissolved oxygen (DO) was measured at the start of the experiment, on duplicate 200-mL samples of hypoxic and normoxic water, using a modified Winkler method in which samples were preserved with prepackaged powdered reagents, then titrated with 0.025 N sodium thiosulfate. All DO reagents were obtained from Hach Company (Loveland, CO). The experiment was conducted at 16°C.

In the second experiment, *C. riparius* larvae were exposed to hypoxic conditions in three mesocosms for four different intervals of 24 h in September, 1995. One cage, containing five larvae, was placed into each mesocosm at the start of each interval. Dissolved oxygen concentrations (DO) and temperature were measured with continuous water quality monitors (AQUA 2000 prototypes, Biodevices, Inc., Ames, IA).

Effect of a pesticide mixture on choiinesterase activity

For exposure to the pesticide mixture, mesocosms were dosed from a common mixing tank, where stock solutions of chlorpyrifos, atrazine, and metolochlor were mixed with well water. (Chlorpyrifos was obtained as 98% powder from DowElanco. Atrazine and metolachlor were obtained from the Ciba-Geigy Corporation.) Nitrate (10 mg L^{-1}) was mixed in the dosing solution to simulate amounts in agricultural drainage. Six mesocosms were treated with the mixture in October, 1995. To investigate effects of *Typha* on the fate of the pesticide mixture, three of the dosed mesocosms were cleared of *Typha* stalks and tubers 14 d prior to treatment. Mesocosms from which macrophytes were removed are referred to as "open-water" mesocosms, and *Typha* -containing mesocosms are referred to as "vegetated."

Temperature and DO were monitored continuously throughout the experiment in four of the six treated mesocosms using water quality monitors (AQUA 2000 prototypes, Biodevices Inc., Ames, IA). Water samples for pesticide

analysis were collected close to the times chironomids were removed, filtered using a GF/F 0.7 μm (Whatman Paper Ltd.), and frozen until analysis. Pesticide concentrations in filtered water samples were analyzed with HPLC by a method modified from Steinheimer (1993).

Three cages of *C. riparius* larvae were placed into each mesocosm as the pesticide mixture was sprayed into the treated mesocosms; these were removed from the mesocosms at 6 h, 18 h and 28.5 h post-dose. At 28.5 h, a single set of larvae was placed in each mesocosm and remained until 42 h; this was repeated again from 42 to 66 h.

A laboratory experiment was performed in October, 1996, to determine whether the herbicides atrazine and metolachlor depressed ChE activity. *C. riparius* larvae were exposed for 19 h to solutions of individual pesticides and to a mixture of all three pesticides (atrazine 50 μg L^{-1}, chlorpyrifos 0.5 μg L^{-1} and metolachlor 50 μg L^{-1}) at 20°C in an environmental chamber. Stock solutions of each pesticide were first made as 10 mg pesticide in 10 mL acetone. The test solutions were made by diluting portions of the stock solutions in 500 mL volumetric flasks with carbon-filtered water. 150 mL of each solution was poured into three beakers, then chironomids were added to the beakers. There were three beakers per treatment, with six larvae per beaker. Controls consisted of 50 μg L^{-1} acetone in carbon-filtered water. Individual larvae were analyzed for ChE activity; results are reported as means for each beaker.

To determine if 50 μg L^{-1} acetone affected survival and ChE activity in chironomids, and if acetone was an appropriate carrier for the mixed pesticides experiment, an earlier experiment was conducted in the same way as the mixed pesticides test just described. Chironomid larvae were exposed for 24 h to 50 μg acetone L^{-1} water in 250 mL beakers; ChE activities were compared to larvae kept in beakers of control water.

Concentrations of atrazine and metolachlor were not analyzed in the laboratory experiment; nominal values are used. Chlorpyrifos concentrations were determined using a commercial kit, RaPID Assay® (Ohmicron, Newtown, PA), in which enzyme linked immunosorbent assay (ELISA) reagents react with individual pesticides to form a blue color; absorbance was determined in a spectrophotometer at 450 nm. Concentrations were determined from a standard curve made from standards provided by the vendor; the standard curve was verified using a chlorpyrifos check standard provided by the vendor. Samples were taken from the chlorpyrifos and mixed pesticides solutions shortly after mixing. Duplicates of each sample were analyzed.

Cholinesterase analysis

ChE activities were assayed on individual *C. riparius* larvae. Each larva was removed from its test container and blotted twice on a Kimwipe before weighing to 0.1 mg on a Sartorius balance. After weighing, each larva was homogenized in pH 7.4 Tris buffer (reagents obtained from Sigma, St. Louis, MO) with a Teflon pestle

and glass homogenizer tube. Dilutions were generally around 100-fold w/v (e.g., a larva weighing 2.5 mg would be homogenized in 0.250 mL Tris pH 7.4 buffer; the dilution factor 101 would be used in the ChE activity calculation).

Larvae used in mesocosm experiments were homogenized immediately and homogenates were kept refrigerated until analysis, which generally occurred within 6 h, or were frozen in liquid nitrogen and analyzed within four weeks. Separate tests showed that ChE activity of homogenates does not change when kept refrigerated for up to 24 h (data not shown). Larvae used in laboratory experiments were wrapped in wax paper after weighing, and immediately frozen at -80°C; these were analyzed within two months. Numerous studies of long-term storage of fish and bird tissues and homogenates have demonstrated that storage at -20°C and below will maintain ChE activity over a period of several weeks (Fairbrother *et al.*, 1991).

Total ChE activity was measured; no differentiation between acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities was made. ChE analysis was done with a colorimetric method, modified to use a THERMOmax microplate reader and SOFTmax software (Molecular Devices Corp., Menlo Park, CA) to monitor the rate of formation of a yellow product, 5-thio-2-nitrobenzoate, from a reaction of 5,5-dithiobis-2-nitrobenzoic acid with thiocholine released by cleavage of acetylthiocholine by ChE (Ellman *et al.* 1961; Hill & Fleming, 1982; Gard & Hooper, 1993). Increase in absorbance at 405 nm was monitored for 2 min at 30°C with readings every 8 s. The software calculated each maximum reaction rate

(Vmax) by doing a series of regressions on the 16 data points. ChE activities are reported as μM acetylthiocholine hydrolyzed min^{-1} g^{-1} tissue for *C. riparius*. Reactions were optimized at 30°C (maintained by an incubator in the microplate reader) and 7.94×10^{-4} M acetylthiocholine for *C. riparius*. The reaction mixture was kept at pH 8.0 by Tris buffer; this pH has been found to be optimal for hydrolyzing acetylthiocholine by *C. riparius* ChE (Detra & Collins, 1986).

Homogenates were analyzed in triplicate; each was vortexed for 10 sec immediately prior to pipetting triplicates into a microplate. Analysis of a homogenate was repeated if the triplicate coefficient of variation (CV) was greater than 15%. With each run, a triplicate of a check standard made from pooled homogenates, and stored in cryovials in liquid nitrogen, was also analyzed. A run was repeated if the check standard CV was greater than 15%. See Appendix A for ChE analysis standard operating procedure and Appendix D for results of chironomid check standard assays.

Statistics

Normal distribution of ChE activity in each experiment was checked using the Shapiro-Wilk statistic, W, in PROC UNIVARIATE (SAS, 1993). Univariate analysis of variance (ANOVA) was used to test for differences among group means. Linear regressions were done on data from hypoxia exposures in PROC REG (SAS, 1993) to look for trends over time. Results were considered significant at an α level of 0.05.

Results

Effect of hypoxia on cholinesterase activity

In the laboratory experiment, DO of the hypoxic water was 1.90 ± 0.02 mg L^{-1}. DO of the normoxic, control water was 7.86 ± 0.04 mg L^{-1}. There was no effect of hypoxia on ChE activity in the laboratory experiment (Figure 2.1). The mean ChE of chironomids in all replicates was 3.12 ± 0.05 μM acetylthiocholine hydrolyzed min^{-1} g^{-1} tissue In a linear regression of mean ChE activity for larvae in each replicate, there was no significant relationship (r^2=0.0011; P=0.92).

Mean water temperatures ranged 7-18°C during the mesocosm experiment. DO was generally 0.1 mg L^{-1}, and increased 1.2 mg L^{-1} in the afternoons. Therefore, chironomid larvae were exposed continuously to severe hypoxia during the experiment (100% saturation at 7-18°C is 12.1-9.5 mg L^{-1}; APHA, 1992).

ChE activities were not affected by exposure to hypoxia in the wetland mesocosms, as in the laboratory experiment. The mean ChE activity of the 24-h exposures was 3.21 ± 0.16 μM acetylthiocholine hydrolyzed min^{-1} g^{-1} tissue.

Effect of a pesticide mixture on cholinesterase activity

Colonies of cyanobacteria were starting to bloom in open-water mesocosms at the start of the mesocosm experiment. Photosynthesis from these colonies visibly supersaturated open-water mesocosms with oxygen throughout each day; bubbles rose from algal mats almost immediately each dawn. This contrasted with conditions in vegetated mesocosms, which maintained lower temperatures and DO.

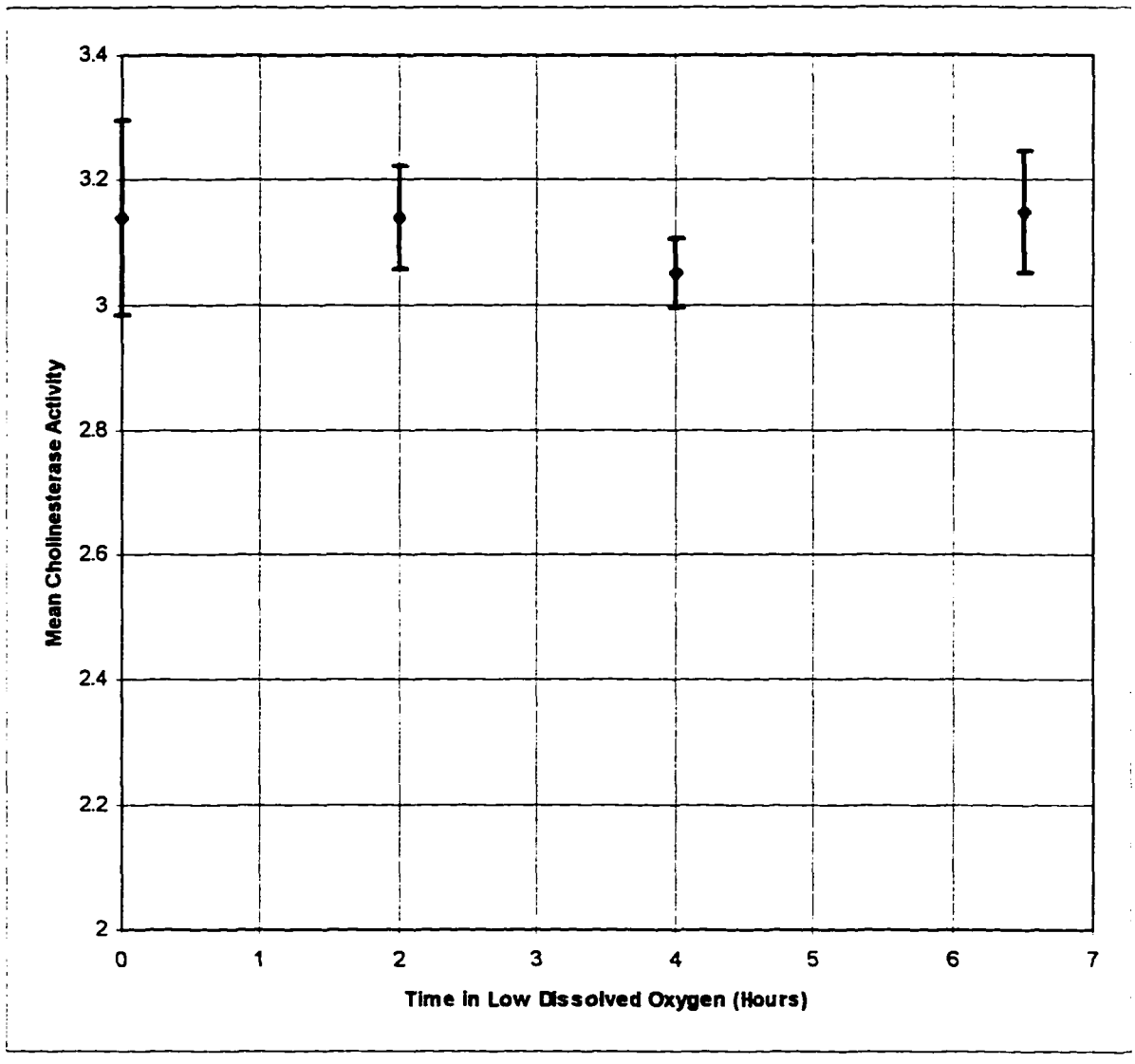

Figure 2.1. Mean cholinesterase activity (μM acetylthiocholine hydrolyzed min^{-1} g^{-1} tissue) vs. time in BOD bottles filled with carbon-filtered water through which nitrogen was bubbled (DO = 1.90 mg L^{-1}) for *Chironomus riparius* larvae. Each mean consists of three replicates, BOD bottles containing four larvae. Controls (time = 0) were in carbon-filtered water (DO = 7.86 mg L^{-1}). Error bars are standard errors of means.

In the two open water mesocosms, DO concentrations ranged from 1 mg L^{-1} to > 20 mg L^{-1}, and temperatures were between 9 and 22°C. In contrast, DO in the three vegetated mesocosms stayed below 2 mg L^{-1}, with temperatures between 5 and 15°C.

Chlorpyrifos concentrations were initially higher in open-water than in vegetated mesocosms during the first 24 h; subsequently, concentrations were similar in all treated mesocosms (Figure 2.2). In contrast, concentrations of both herbicides were higher in vegetated than in open-water mesocosms during the first 10 d (Figures 2.3-2.4). Mean chlorpyrifos concentrations in both vegetated and open-water mesocosms were greater than 0.5 μg L^{-1} thirteen days after the dose was administered; mean herbicide concentrations were greater than 10 μg L^{-1} at that time.

ChE activity was inhibited in larvae caged in treated mesocosms (Figure 2.5). ChE inhibition was evident 6 h post-dose, when ChE activity in open-water mesocosms averaged 0.20 μM acetylthiocholine hydrolyzed min^{-1} g^{-1} tissue, while mean ChE activity in vegetated mesocosms was 2.30 μM acetylthiocholine hydrolyzed min^{-1} g^{-1} tissue. Mean ChE activity at 28.5 h post-dose was 0.15 μM acetylthiocholine hydrolyzed min^{-1} g^{-1} tissue in open-water and 0.13 μM acetylthiocholine hydrolyzed min^{-1} g^{-1} tissue in vegetated mesocosms; these were the last of the larvae placed in mesocosms at the time of the dose. Larvae placed in mesocosms at 28.5 h and removed 42 h post-dose, had mean ChE activities of

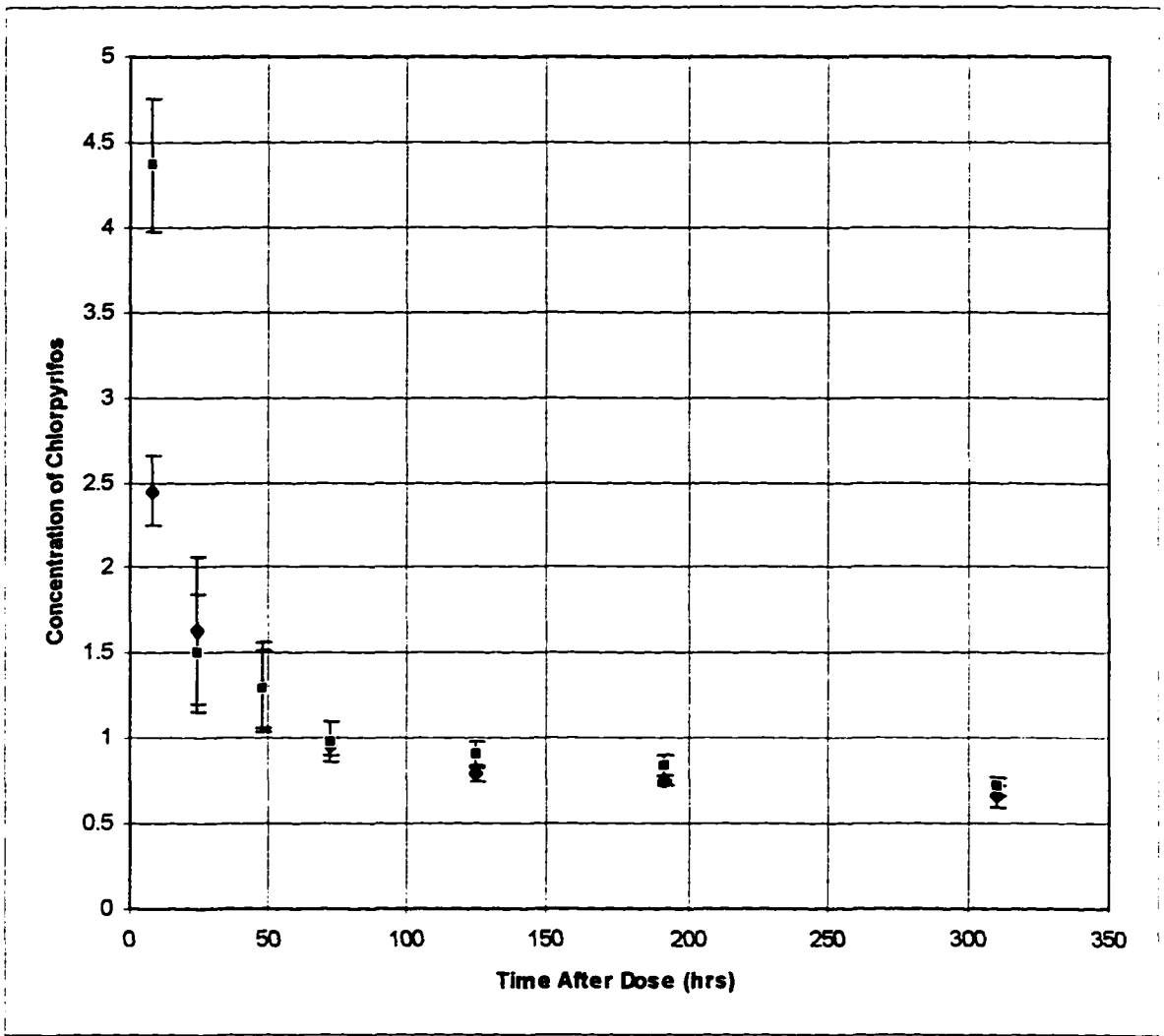

Figure 2.2. Chlorpyrifos concentrations ($\mu g\ L^{-1}$) in mesocosms at several times after application of dose. Diamonds are means of vegetated mesocosms, squares are means of open-water mesocosms. Each mean represents three mesocosms. Error bars are standard deviations.

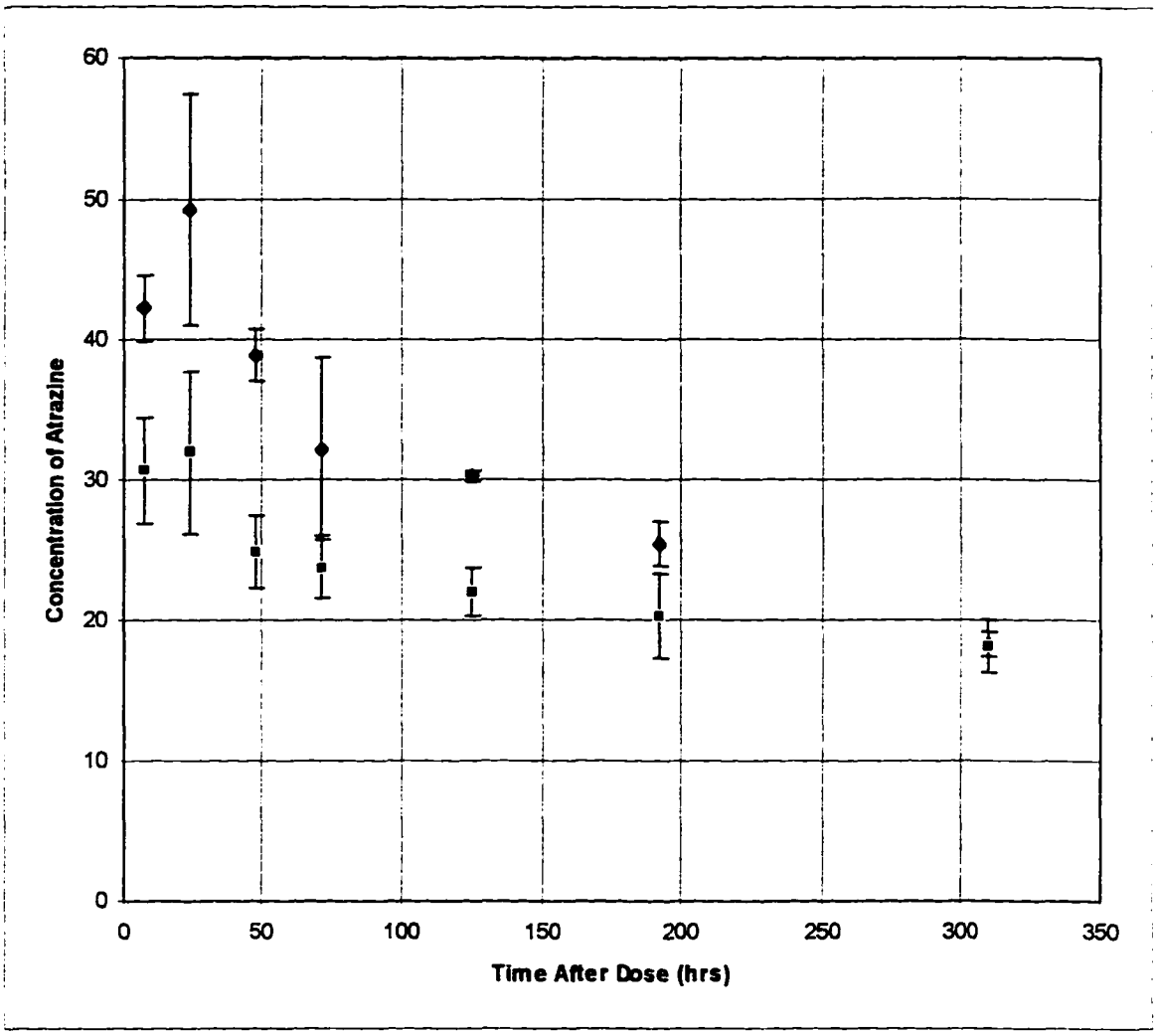

Figure 2.3. Atrazine concentrations ($\mu g\ L^{-1}$) in mesocosms at several times after application of dose. Diamonds are means of vegetated mesocosms, squares are means of open-water mesocosms. Each mean represents three mesocosms. Error bars are standard deviations.

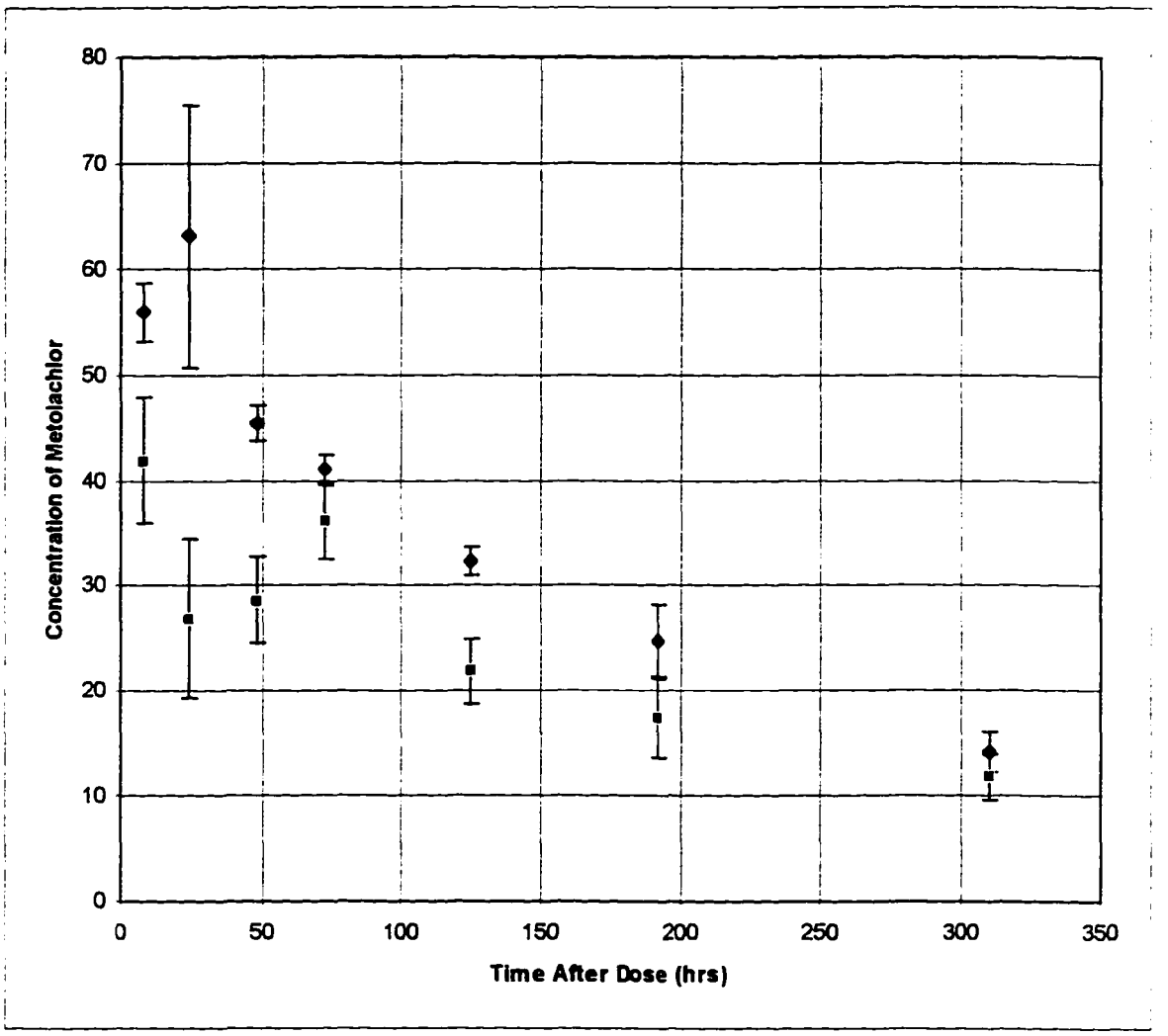

Figure 2.4. Metolachlor concentrations ($\mu g\ L^{-1}$) in mesocosms at several times after application of dose. Diamonds are means of vegetated mesocosms, squares are means of open-water mesocosms. Each mean represents three mesocosms. Error bars are standard deviations.

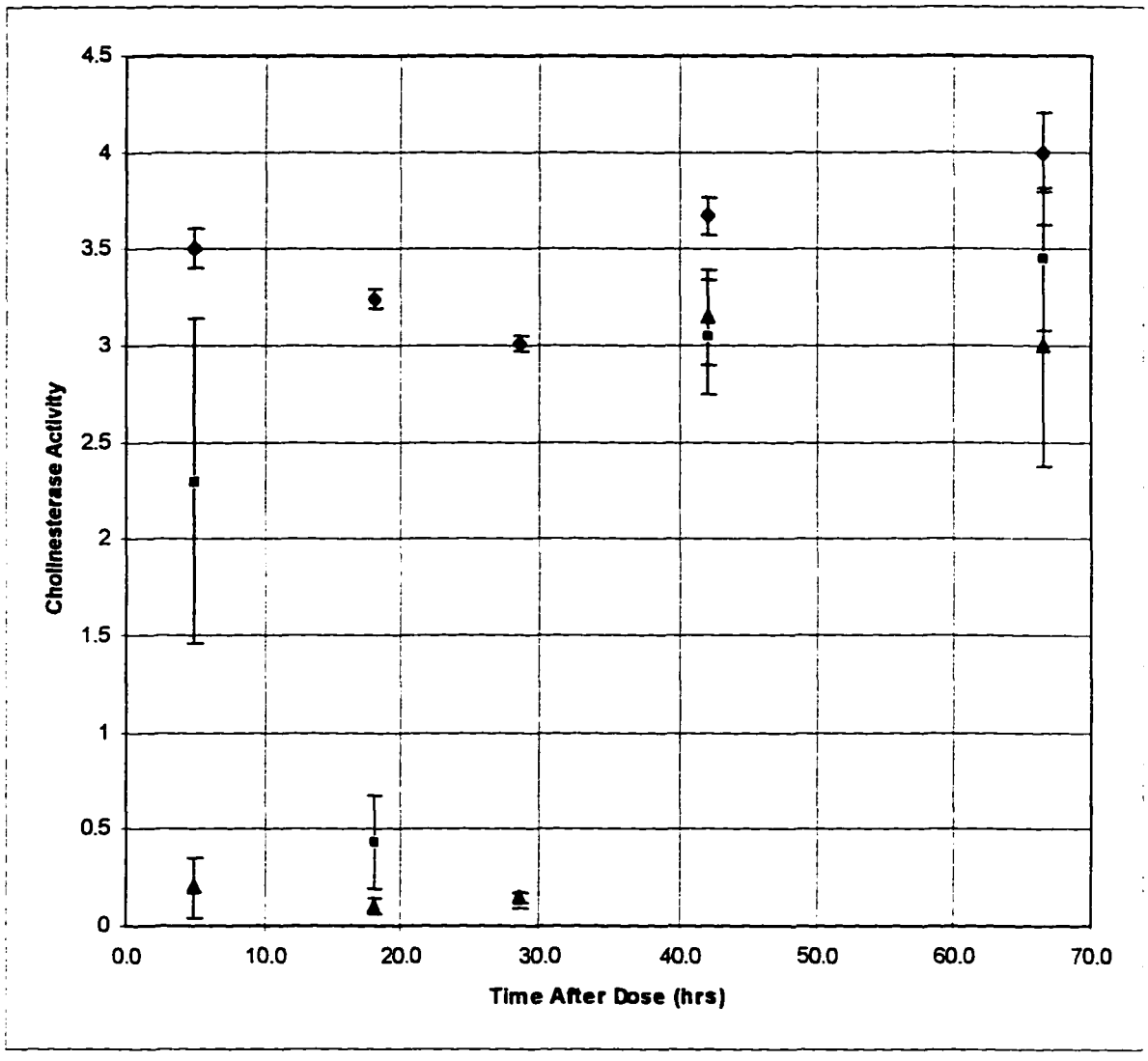

Figure 2.5. Cholinesterase activity (μM acetylthiocholine hydrolyzed min^{-1} g^{-1} tissue) of larval *Chironomus riparius* vs. time after dosing of wetland mesocosms with a pesticide mixture of chlorpyrifos, atrazine and metolachlor. Diamonds are means of controls, squares are means of vegetated mesocosms, triangles are means of open-water mesocosms. Each mean represents one sample cell containing 3 larvae. Error bars are standard deviations.

3.15 and 3.05 μM acetylthiocholine hydrolyzed min^{-1} g^{-1} tissue. This indicates that effects of the pesticide dose were negligible just over a day later. There was no significant difference in ChE activity between larvae caged in treated and control mesocosms at 42 h and removed at 66 h.

In the laboratory experiment comparing acetone and water-only exposures, there was no difference between mean ChE activities. The mean ChE activity for larvae exposed to acetone was 2.49 ± 0.14 μM acetylthiocholine hydrolyzed min^{-1} g^{-1} tissue, while the mean ChE activity for water-only exposures was 2.55 ± 0.09 μM acetylthiocholine hydrolyzed min^{-1} g^{-1} tissue. Therefore, acetone was an acceptable choice for carrier in the mixed pesticides experiment.

In the mixed pesticide laboratory experiment, chlorpyrifos concentrations measured in samples taken from the chlorpyrifos and mixed pesticides solutions shortly after mixing were 0.31 ± 0.06 and 0.48 ± 0.08 μg L^{-1}, respectively. All chironomids in beakers containing chlorpyrifos were in tetany and appeared "shriveled" at the end of the test, while larvae in controls and in atrazine- and metolachlor-only treatments all appeared normal in size and activity. There were no significant differences in ChE activity between larvae exposed to either atrazine or metolachlor and controls (Figure 2.6). There was no significant difference between larvae exposed to chlorpyrifos alone and the pesticide mixture.

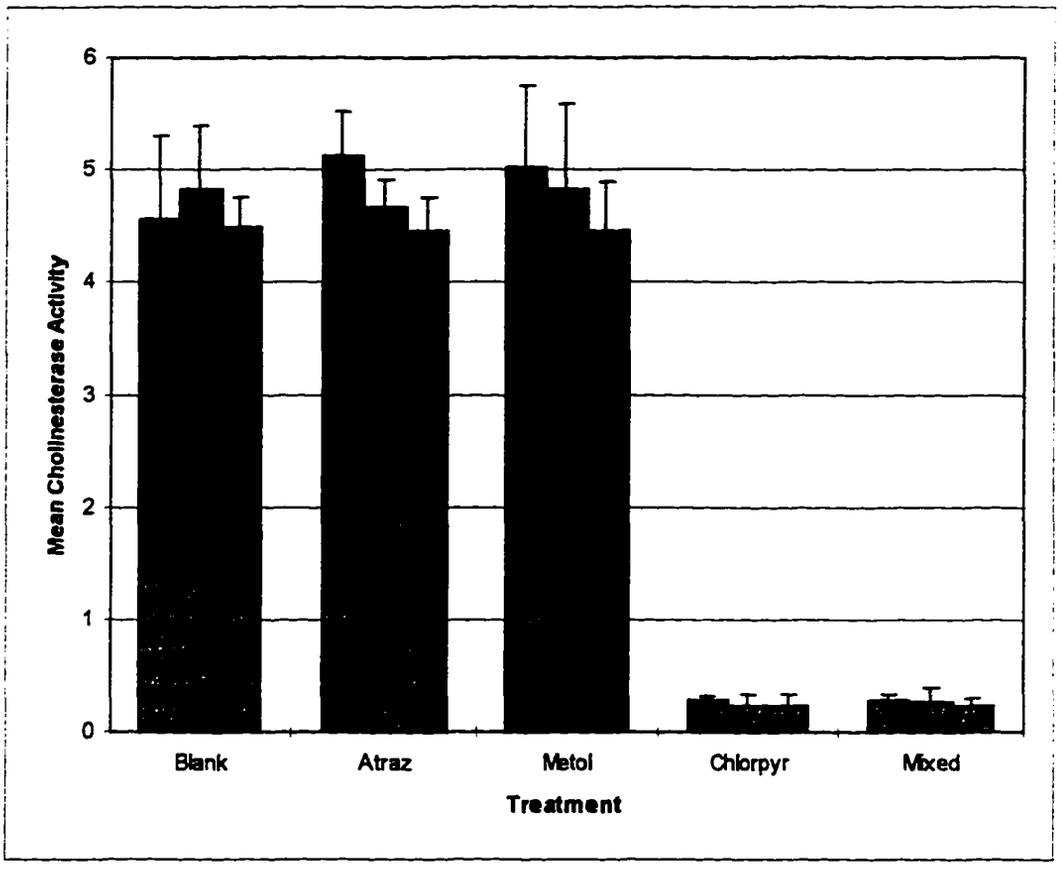

Figure 2.6. Mean cholinesterase activity of larval *Chironomus riparius* exposed for 19 h to pesticide solutions in the laboratory. Treatments are as follows: control (acetone 50 μg L^{-1}), atrazine (50 μg L^{-1}), metolachlor (50 μg L^{-1}), chlorpyrifos (0.5 μg L^{-1}), and a mixture of atrazine, metolachlor and chlorpyrifos (50, 50 and 0.5 , μg L^{-1} respectively). Each column represents a replicate of six larvae. Error bars are standard deviations.

Discussion

Effect of hypoxia on cholinesterase activity

ChE activity in larval chironomids was not decreased in response to DO concentrations of 1.9 mg L^{-1} and below. Hypoxia was chosen for investigation because it is an important stressor in wetlands. Although chironomids are able to tolerate low DO concentrations (Augenfeld, 1967; Frank, 1983; Bairlein, 1989; Heinis & Crommentuijn, 1992), there is a cost for adapting to this stressor (Nagell & Landahl, 1978). Seldom has ChE activity been determined for organisms under low oxygen conditions, or has potential correlation between DO and ChE activity been investigated. Freshwater mussels collected from North Carolina streams having DO from 3.8 to 13 mg L^{-1} had highly variable ChE activities, but the variation was not significantly correlated with DO (Varela & Augspurger, 1996). Also, ChE activity was not inhibited in rainbow trout exposed to 3 mg L^{-1} for 1 h (Hoy *et al.*, 1991). The lack of ChE inhibition in chironomids under hypoxic conditions in the present study is in agreement with these results, and indicates that hypoxic conditions would not affect the use of ChE activity as a biomarker for OP exposure.

Effects of a pesticide mixture on cholinesterase activity

Neither atrazine nor metolachlor appeared to potentiate ChE inhibition by chlorpyrifos. If there is any synergism between these compounds, a possibility suggested by the increased lethality of parathion to mosquito larvae when mixed

with atrazine (Liang & Lichtenstein, 1974), that synergism does not involve ChE inhibition.

Interestingly, a greater ChE inhibition was seen at chlorpyrifos concentrations less than 0.5 $\mu g\ L^{-1}$ in the laboratory experiment, than at twice that concentration in the mesocosms. There are several explanations possible for this apparent discrepancy, including the use of different analytical methods, different temperatures between the experiments, and differences in bioavailability. Different methods were used to measure chlorpyrifos concentrations, i.e., HPLC for concentrations in mesocosms and ELISA for concentrations in the laboratory. These two methods have been compared for analysis of chlorpyrifos-ethyl in two types of water samples; results were highly correlated (Oubina *et al.*, 1996). Results of the ELISA method for chlorpyrifos have been confirmed using GC (Lawruk *et al.*, 1996). Therefore, it seems unlikely that the differences in method would have given such large differences in measured chlorpyrifos.

A second potential explanation for unexpected differences in ChE inhibition is the difference in temperature between the experiments. Water temperatures in the mesocosm experiment ranged 9-22°C in open-water mesocosms, and 5-15°C in vegetated ones. The lowest temperatures occurred during the first three days of the experiment, when chironomids were caged in the mesocosms. In contrast, the laboratory experiment was conducted at 20°C. The toxicity of one OP, parathion, has been shown to increase considerably over this temperature range. The toxicity of parathion to *C. riparius* at pH 6 increased nearly 10-fold, with the 24-h LC_{50}

decreasing from 60.0 to 6.9 μg L^{-1}, when the temperature was raised from 10°C to 20°C (Lydy *et al.*, 1990). Lydy *et al.* (1990) attributed the increased toxicity to increased metabolic conversion of parathion to paraoxon. Paraoxon and chlorpyrifos oxon are the forms of the insecticides that cause ChE inhibition, as well as toxicity. If increased metabolic activation of chlorpyrifos occurs with increasing temperature, greater ChE inhibition could occur within a shorter time and at lower concentrations at 20°C than at 9°C. To compensate for such a temperature effect, chironomids could be caged in mesocosms for longer exposures at lower temperatures.

Finally, chlorpyrifos may have been more bioavailable to the chironomids in the laboratory experiment than in the mesocosm experiment. In the mesocosms there was much suspended organic matter for chlorpyrifos to adsorb to; some of this organic matter may have passed through the 0.7 μm filter and then dissolved during subsequent extraction. Hence, chlorpyrifos may have been measured that was not bioavailable to the chironomids.

Another difference between the lab and mesocosm experiments was the addition of nitrate (10 mg L^{-1}) which was added to mesocosms along with the pesticide mixture. Nitrate in that concentration was not anticipated to affect ChE activity: an *in vitro* assay of $NaNO_3$ found only "slight effect" on ChE activity at 620 mg L^{-1} nitrate (Olson & Christensen, 1980). ChE activity should be determined in intact chironomids exposed to nitrate to confirm that there is no effect, however.

Atrazine and metolachlor were chosen for investigation of potential interactions with chlorpyrifos because both are widely used herbicides (ERS, 1994). Exposure of aquatic insects to OPs would generally occur along with exposure to other pesticides. Potential synergism in toxicity between atrazine and ChE-inhibiting insecticides has been investigated in aquatic systems, with mixed results. Atrazine mixed in water with the OP, parathion, was shown to synergize toxicity of parathion to third-instar larvae of the mosquito, *Aedes aegypti* (Liang & Lichtenstein, 1974). While atrazine (10 mg L^{-1}) was not toxic alone and parathion (15 μg L^{-1}) caused 20% larval mortality, a mixture of the two pesticides in the same concentrations caused 73% mortality. On the other hand, no synergism was found between atrazine and the carbamate insecticide, carbofuran, when *C. tentans* larvae were exposed to both in a series of concentrations (Douglas *et al.*, 1993). No studies described in the literature investigated potential synergistic effects on ChE activity. Results from the present study indicate that no such synergism occurs.

This study has shown that caged larval chironomids placed in shallow wetland mesocosms could be used to detect an influx of a ChE-inhibiting pesticide, without interference from concurrently-dosed herbicides or hypoxia. Caged amphipods have been used to detect contamination by malathion below watercress beds (Crane *et al.*, 1995); as with the present study, caged animals were placed directly in the path of inflowing insecticide. Monitoring may not work as well under other conditions. For example, in a deeper body of water, the pesticide may be

diluted substantially before reaching animals. It seems extremely unlikely that profundal benthos would be effective for this type of monitoring.

A potential drawback to using field-collected (uncaged) animals for monitoring is that, especially with chironomids, keying an organism to species before measuring its ChE activity is not feasible because of the keying method required (Webb & Scholl, 1985). ChE activities, and optimal substrate concentrations for measuring, can vary considerably across even congeneric species in birds (Hill, 1988). It is unclear whether there are similar differences for aquatic invertebrates; until this concern is resolved, it is best to use known species taken from a laboratory culture.

As with previous studies of ChE activity, in this study high individual variation was seen. Such variation can impede diagnosis of exposure to ChE inhibitors in wild populations (Fleming *et al.*, 1995). In fact, after determining ChE activity in several aquatic invertebrates, Day and Scott (1990) questioned whether ChE inhibition could be used to monitor sublethal exposures; however, others (e.g., Detra & Collins, 1991; Kuhn & Streit, 1994) have had more success. The results of the present study suggest that ChE activity in caged chironomids is effective as a biomarker of OP exposure. Although ChE inhibition was less sensitive than HPLC for detection of chlorpyrifos in wetland mesocosms, caging of chironomids for longer intervals may allow detection of lower OP concentrations than in the present study, perhaps at or even below the detection limit of HPLC, and providing a less expensive and rapid technique for monitoring exposure of wetlands to OPs.

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GENERAL CONCLUSIONS

Monitoring of OP contamination in aquatic systems using ChE activity in organisms taken from those systems is effective when the organisms are directly exposed to OP influx. When chironomids were placed in wetland mesocosms dosed with chlorpyrifos in the present study, for example, ChE activity was significantly inhibited. ChE inhibition has also been documented in the literature when organisms are caged in the path of inflows containing OPs (Crane et al., 1995), or when fish were collected from rivers contaminated by effluents of pesticide manufacturers (Williams and Sova, 1966; Coppage and Braidech, 1976). To improve the efficacy of monitoring for OP contamination in aquatic systems, caged organisms are preferred over collection of wild-caught animals. Caged animals can be placed in locations where contamination is most anticipated, and greater control of exposures can be maintained. Other advantages of animal reared in the laboratory over wild-caught animals include knowledge of health status and organisms' histories, and knowledge of species (especially important with animals like chironomids, which are difficult to identify to species).

Results in the present study of monitoring bluegill from the Mark Twain National Wildlife Refuge showed no evidence of cholinesterase inhibition on any of the sampling dates. It seems likely that ChE inhibitors did not reach the backwater areas in sufficient concentrations to cause detectable ChE inhibition in bluegill collected from those areas.

High variation in ChE activity was found in the present study. Bluegill brain ChE activity means ranged 8.25-12.85 μM substrate hydrolyzed/min/g brain tissue. Mean ChE activities for chironomids not exposed to chlorpyrifos ranged 2.5-5.9 μM substrate hydrolyzed/min/g tissue. Several potential causes of variation were investigated in the present study. Of these, sex, temperature differences in the range of 13-26°C, and euthanasia methods did not affect brain ChE activity in bluegill. Exposure of chironomid larvae to hypoxia or herbicides did not affect ChE activity.

In bluegill, ChE activity was inversely related to total length. This result agrees with results of similar comparisons in the literature (Weiss, 1961; Rath and Misra, 1980; Zinkl et al., 1987). In any monitoring using ChE activity, similarly-sized organisms should be used.

Recommendations for future research

Several factors which may potentially affect ChE activity were investigated in this study. Environmental factors including dissolved oxygen, water temperature, and concurrent exposure to two herbicides did not alter ChE activity. Characteristics of individual animals including sex and size were investigated; while sex did not affect ChE activity, size did. There are other aspects of these or related factors which have the potential to significantly affect ChE activity, which should be investigated.

Dissolved oxygen is only one water quality factor that may potentially affect ChE activity. Other water quality factors that may be of concern in animals raised in crowded laboratory cultures are ammonia and nitrite concentrations. Chironomid larvae taken from cultures having total ammonia nitrogen concentrations in excess of 0.1 mg/L had ChE activities as much as 50% lower than chironomid larvae from cultures with lower ammonia concentrations (data not shown). Although ammonia did not affect freshwater mussel ChE activity in normal environmental concentrations (Varela and Augspurger, 1996), nor did ammonia decrease fathead minnow muscle ChE activity in an in vitro laboratory test in concentrations up to 0.01 M (Olson and Christensen, 1980), ammonia may be a factor in ChE variation in higher concentrations or over longer durations, as may be present in crowded chironomid cultures. It is possible that ammonium cation (NH_4^+) is a ChE inhibitor even if un-ionized ammonia (NH_3) is not. Several monovalent and divalent cations have been shown to alter ChE activity (Detra and Collins, 1986). Potential effects of ammonium ion, as well as nitrite, a product of ammonia oxidation by bacteria in aquatic systems, should be investigated in both acute and chronic exposures.

Water temperature did not affect brain ChE activity in bluegill in the present study. However, Hogan (1970) did see a substantial difference in ChE activity that may have been either related to water temperatures, such that bluegill collected at temperatures near the freezing point may have significantly lower ChE activities, or the lower ChE activities may have been associated with less feeding or hormonal changes that occur in winter in temperate climates. If bluegill were acclimated to

low temperatures in laboratory experiments, the cause of decreased ChE activities in winter could be determined.

Investigations into potential synergism of ChE inhibition by chemicals in mixtures is important. Herbicides other than atrazine and metolachlor should be considered. But as those who monitor presence of herbicides in aquatic systems by chemical methods have shown, degradation products can be present in appreciable quantities as well, and often have quite different chemical properties from the parent compounds (Thurman et al., 1992). Potential effects of degradation compounds should not be overlooked. Newer insecticides that may also affect ChE activity, including pyrethroids and insect growth regulators, should be investigated. Some inorganic contaminants, such as the metals, cadmium, copper, lead and mercury, are known ChE inhibitors. Synergism between copper and the OP, methidathion, has been demonstrated (Flammarion et al., 1996). Other inorganic compounds should be investigated. Organic compounds, such as such as crude oil, have been shown to be ChE inhibitors (Chambers et al., 1979). There are many other candidates for investigation.

Finally, effects of ChE inhibitors generally are studied under conditions where exposures last several hours to several days. But in many aquatic systems, exposures are pulsed over much shorter intervals. Much higher concentrations of ChE inhibitors would be required to cause effects in pulsed exposures than in exposures of longer duration. Both inhibition and recovery of ChE activity should be studied under pulse conditions.

APPENDIX A. STANDARD OPERATING PROCEDURE FOR THE DETERMINATION OF CHOLINESTERASE ACTIVITY

I. Introduction

Cholinesterase activity is a measure of the amount of active cholinesterase in tissues. Cholinesterase (ChE) is a group of enzymes, which have a known function in hydrolysis of the neurotransmitter, acetylcholine. The determination of ChE activity can be used as a biomarker to determine if organisms have been exposed to organophosphorus or carbamate insecticides; both types of insecticides inhibit ChE activity as a primary mode of action.

A spectrophotometric assay using a plate reader is used to determine ChE activity in fish brain tissue (Ellman *et al.*, 1961; Hill and Fleming, 1982; Corvallis Environmental Research Laboratory, 1987; The Institute for Wildlife and Environmental Toxicology, 1991). ChE activity is determined from the result of two reactions occurring in the assay solution: acetylthiocholine hydrolysis and reaction of the thiocholine product with a colorimetric reagent. The assay solution consists of an aliquant from the homogenized brain sample (source of the ChE enzyme), acetylthiocholine (AThCh substrate), and 5,5-dithiobis(2-nitrobenzoic acid) (DTNB colorimetric reagent).

AThCh is an analogue of the natural ChE substrate, acetylcholine, in which a sulfur atom replaces the esteric oxygen of acetylcholine. Hydrolysis of AThCh results in the formation of a negatively charged thiocholine complex and an acetate ion. This thiocholine complex reacts with DTNB to generate a stable, yellow-colored anion (5-thio-2-nitrobenzoate) which absorbs light strongly at 412 nm. The rate of formation of the yellow-colored anion can be measured and subsequent calculations can determine the ChE activity for the sample.

This assay has been optimized for bluegill (*Lepomis macrochirus*) and one species of chironomid (*Chironomus riparius*).

II. Materials

A. Chemicals (all of these may be purchased from Sigma Chemical Co.)

1. Acetylthiocholine iodide (AThCh)
2. 5,5-dithiobis-2-nitrobenzoic acid (DTNB)
3. Sodium bicarbonate, Reagent Grade
4. Trizma 7.4 pH pre-set crystals
5. Trizma 8.0 pH pre-set crystals
6. 1.0 N HCl
7. 1.0 N NaOH

B. Equipment

1. Spectrophotometer: THERMOmax automated kinetic microplate reader (Molecular Devices Corporation, Menlo Park, CA) interfaced with a desk top computer (Zenith z-386/20). SOFTmax software (Molecular Devices Corporation, Menlo Park, CA) on the computer controls the THERMOmax reader and enclosed incubator to hold reactions at optimal temperatures.

a. Bluegill: 25°C.

b. Chironomids: 30°C.

2. Constant temperature water bath set at same temperature as incubator (Precision Scientific Co., Chicago).

3. Two small ice chests half-filled with crushed ice: one for Tris 7.4 buffer, DTNB, and AChTh reagents, the other for homogenates. Alternately, homogenates may be kept in refrigerator.

4. Disposable test tubes (13x100 mm), enough to dilute samples + 2 for DTNB and AThCh solutions (solutions are easier to pipette out of disposable test tubes than out of volumetric flasks).

5. Multi-aliquot, variable volume pipette (e.g., Eppendorf Combitip Pipette) with disposable tips 10µl, 50 µl, 100 µl and 1000 µl (e.g., Eppendorf Combitips).

6. Single aliquot, variable volume pipette, 100-1000 µl range, with disposable tips for diluting homogenates.

7. Vortex mixer for mixing homogenates immediately before analysis.

8. Magnetic stirrer and stir bars for use during Tris buffer titration.

9. pH meter (e.g., Corning pH/ion meter 150, Scientific Instruments, Corning Glass Works, Medfield, MA). Calibrate with pH 7.00 and pH 10.00 buffers.

10. 96 multi-well microplates, non-sterile, flat-bottomed (Dynatech Microtiter Plates, Cat. No. 001-012-9205, Dynatech Labs, Chantilly, VA 800-336-4543).

11. Analytical balance, sensitive to 0.1 mg (e.g., Sartorius A200S, Sartorius Corp., Bohemia, NY).

12. Motorized stirrer (e.g., Tri-R Stir-R, Model K41, 500-11000 RPM, 1/15 HP, 115 V, 50/60 CY, Rockville Centre, NY). Teflon pestle and 15 mL glass homogenizer tubes (both made by Glas-Col, Terre Haute, IN).

13. Volumetric flasks, 5-50 mL and 1000 mL.

14. Weigh boats, glass (for DTNB) and plastic (for other reagents, samples).

15. Chemical spatulas.

16. Dissection scissors, 4-1/2", 1 each straight and curved, for bluegill dissection (e.g., Cat. No. K3-62-1810 and K3-62-1830, Carolina Biological Supply Co., Burlington, NC). Use microdissection scissors for fish less than ~5 cm total length (e.g., Cat. No. K3-62-3555, Carolina Biological Supply Co., Burlington, NC).

17. Microdissection or watchmaker's forceps with extra fine points, for transfer of chironomids (e.g. Cat. No. K3-62-4734 or K3-62-4791, Carolina Biological Supply Co., Burlington, NC).

III. Preparation of buffers, reagents and substrate.

Nanopure or distilled water is used to mix solutions. Flasks containing solutions are labeled with chemical name, date, and preparer's name. Solutions are prepared according to the following procedures:

A. Tris 7.4 pH buffer solution

1. Weigh 7.58 g Trizma 7.4 pre-set crystals in a weigh boat and transfer to a 1-liter volumetric flask.

2. Make a quantitative transfer of chemical by rinsing the weigh boat with water at least 3 times, until no crystals are seen in weigh boat.

3. Dilute to the mark with water. Stopper flask or cover with Parafilm and invert flask 20 times to mix.

4. Check pH and adjust to pH 7.40 with HCl or NaOH.

5. Store in the refrigerator (4°C). Buffer solution will be good for three weeks. Before each use, check pH and adjust to pH 7.40 with HCl or NaOH.

B. Tris 8.0 pH buffer solution

1. Weigh 8.02 g Trizma 8.0 pre-set crystals in a weigh boat and transfer to a 1-liter volumetric flask.

2. Make a quantitative transfer of chemical by rinsing the weigh boat with water at least 3 times, until no crystals are seen in weigh boat.

3. Dilute to the mark with water. Stopper flask or cover with Parafilm and invert flask 20 times to mix.

4. Check pH and adjust to pH 8.00 with HCl or NaOH.

5. Store in the refrigerator (4°C). Buffer solution will be good for three weeks. Before each use, check pH and adjust to pH 8.00 with HCl or NaOH.

C. AThCh substrate

(NOTE: AThCH must be optimized for each species according to a procedure as described in TIWET SOP 202-16-01. Each time work is begun with a new species, optimize first!)

1. Weigh out acetylthiocholine iodide crystals. Crystals are stored in the freezer.

a. For bluegill adults and juveniles, the optimal substrate concentration has been determined to be $1 \times 10^{-2.6}$ M (3.16×10^{-3} M). Weigh 0.0605 g AThCh in a weigh boat and transfer to a 10 mL volumetric flask.

b. For *Chironomus riparius* larvae, the optimal substrate concentration is $1 \times 10^{-3.1}$ M (7.94×10^{-4} M). Weigh 0.0191 g AThCh in a weigh boat and transfer to a 10 mL volumetric flask.

2. Make a quantitative transfer of crystals by rinsing weigh boat with water at least 3 times, until no crystals are seen in weigh boat.

3. Dilute to the mark with water. Stopper flask or cover with Parafilm and invert flask 20 times to mix.

4. Transfer to a labeled amber bottle, or cover flask with aluminum foil and store in the refrigerator (4°C). Substrate solution will be good for up to 3 days.

D. DTNB reagent

1. Weigh 0.0161 g of DTNB in a glass weigh boat and transfer to a 10 mL volumetric flask.

2. Make a quantitative transfer of crystals by rinsing weigh boat with Tris 7.4 pH buffer solution at least 3 times, until no crystals are seen in weigh boat.

3. Weigh 0.0152 g sodium bicarbonate in a glass weigh boat and transfer to the same amber bottle. Again, make a quantitative transfer of crystals by rinsing weigh boat with Tris 7.4 pH buffer solution at least 3 times, until no crystals are seen in weigh boat.

4. Dilute to the mark with buffer solution and mix until dissolved. Store in the refrigerator (4°C). Make up fresh daily.

IV. Analysis procedure:

1. Turn on water bath $\geq$ 1 h prior to analysis, set to temperature (see **B.1.**).

2. Place appropriate volume of Tris 8 pH buffer in water bath (125 mL is usually about right). If the buffer is cold (4°C), allow enough time in water bath for it to come to temperature.

3. Turn on the spectrophotometer (THERMOmax) and control computer. Run the controlling software (double click the SOFTmax icon). Turn the incubator on and set the temperature (see **B.1.**) in the **control** menu. Open the appropriate file (bgche) with the analysis parameters as listed below.

- **a.** wavelength: 405 nm
- **b.** run time: 2:00 min
- **c.** read interval: 8 s
- **d.** OD limit: 0.500 OD
- **e.** lag time: 0.00 s
- **f.** auto mix ON

4. Remove check standards from liquid nitrogen freezer and place in ice or refrigerator to thaw (procedure for making check standard is in **VI.**).

5. Prepare sample homogenates.

a. Bluegill: Transfer fish to refrigerator for partial thawing; fish will be somewhat flexible, but still have ice crystals on surface when it has adequately thawed. This will give brain tissue that is soft enough to distinguish from skull; if fish is thawed too much, brain tissue will liquify. Remove brain tissue by cutting away the top of the skull, severing the optic nerves and then lifting out the brain. Keep the brain tissue in iced pH 7.4 Tris buffer until analysis. Homogenize tissue in pH 7.4 Tris buffer with a motorized Teflon pestle and glass tube. Dilute tissue homogenate using Tris 7.4 pH to an activity appropriate for the spectrophotometer (usually at least 100-fold). Record the fish size data on form #1 and the weights of the brain tissue and appropriate dilutions on form #2. Fish are dissected two at a time, with a control weigh boat of buffer weighed at the beginning and end of each pair. If the weight change in these control weigh boats is $\geq$ 10% of brain weight, then the sample is rejected.

b. Chironomids: Transfer each larva to a kimwipe to blot off excess water, weigh in weigh boat, record weight. Place in homogenizer tube, add enough Tris 7.4 buffer to give a 101-fold dilution (e.g., for a larva weighing 0.0035 g add 350 µL buffer; minimum volume 250 µL buffer), homogenize immediately.

6. Prepare cholinesterase assay plate reader set-up form (#3) indicating the positions of the various samples and check standards and their respective dilution factors. **Template setup** menu in SOFTmax will tell microplate reader which cells to read and how to report results.

7. Mark microplate to indicate where particular samples will be placed.

8. Pipette appropriate amounts of reagent into each well for each determination to be performed. Place the DTNB and AThCh on ice next to the analysis station. All samples should be assayed in triplicate.

Volumes of reagents for the various wells are as follows (in µl):

	Blank	ChE
Tris 8.0 pH	200	170
DTNB	20	20
Enzyme	0	30
AThCh	30	30

9. Add compounds to wells in the order shown in the table. Once the AThCh is added the reaction begins. Immediately select **read** under the **control** heading in the software. The drawer will then open for a few seconds to allow for locking of the plate into place. (Each analysis after the first of the day, the software will prompt and ask if you want to change the existing data file. The default to this question is yes, but click on **no** in order to save each run under its own filename.)

10. After the analysis is complete, type in comments on the data screen and save the file under an appropriate name. Print off a hardcopy of the file.

11. Check the data for any signs of error. Samples with a coefficient of variance (CV) $\geq$ 10 % should be rerun. Also check if the check standards are in control. If the CV of the check standard is $\geq$ 10 %, then the entire run should be repeated.

12. Convert mOD output units into international units of enzyme activity using the following equation:

(((enzyme mOD/min)-(blank mOD/min))/1000) x 0.817 x dilution factor = (µmoles AThCh hydrolyzed/min) / gram tissue.

The above equation is derived from Ellman et al. (1961).

V. Glassware Washing

1. Discard contents, rinse with tap water.

2. Scrub with Alconox detergent.

3. Rinse once with tap water.

4. Rinse 7 times with D.I. water ("blue line"; i.e., tubing is blue).

5. Rinse 7 times with nanopure, resistance $\geq$ 17.0 (change cartridges if resistance drops below 17.0).

6. Leave on counter or place in drying oven. When dry, use or put away.

VI. Preparation and use of check standards

A. Purpose. The purpose of these check standards is to verify the analytical procedure from pipetting of reagents into microplate wells through incubation. Pipetting precision, reagents, and instrument function are checked by use of these check standards; the steps through sample homogenization are not.

B. Use. A triplicate of check standard is pipetted into microplate wells as are the sample homogenates in each run. The mean Vmax should be fairly close to previous runs (a control chart may be used to quantitatively follow run means), and the CV less than 10% (for bluegill; 15% for chironomid check standards). If the mean Vmax differs from the expected range, determine the cause before continuing. If the check standard CV $\geq$ 10% or 15%, repeat the run.

C. Preparation from pooled samples (bluegill). This works well for bluegill but not for chironomid homogenates, as there is seldom excess homogenate beyond what is needed for analysis. Portions of several, well-mixed homogenates may be poured into a homogenizer tube and vortexed for 10 sec to mix. After mixing, analyze as usual. If the Vmax is within the target range (~100 $\pm$ 30 mOD/min for bluegill; ~50 $\pm$ 20 mOD/min is about the highest that can be achieved for chironomids), then pipet ~500-1000 µL into each of 24-36 cryovials, store in liquid nitrogen freezer.

D. Preparation of chironomid check standards. Transfer at least 20-30 chironomids (depending on size) to kimwipe to blot off excess water, then put chrinomids into a tared weigh boat. Get an approximate weight of the group of chironomids, then transfer to a homogenizer tube along with enough Tris 7.4 buffer to make ~100-fold dilution. Homogenize, then analyze as usual. If the Vmax is within the target range (~100 $\pm$ 30 mOD/min for bluegill; ~50 $\pm$ 20 mOD/min is about the highest that can be achieved for chironomids), then pipet ~500-1000 µL into each of 24-36 cryovials, store in liquid nitrogen freezer.

VII. Acknowledgments

This Standard Operating Procedure draws heavily from those written at TIWET (SOP 202-06-03, effective date March 1, 1991, and SOP 202-16-01, effective date February 15, 1991) and by Cole (1995). The glassware washing procedure is modified from one written by T. Stafford (SOP #020, June 14, 1994).

VIII. References

Cole, K.J. 1995. Development of a cholinesterase inhibition assay for monitoring organophosphorus insecticide exposure and effects on bluegill (*Lepomis macrochirus*). Master's thesis, Iowa State University.

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Ellman, G.L., K.D. Courtney, V. Andres, Jr., and R.M. Featherstone. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7:88-95.

Hill, E.F., and W.J. Fleming. 1982. Anticholinesterase poisoning of birds: field monitoring and diagnosis of acute poisoning. Environ. Toxicol. Chem. 1:27-38.

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APPENDIX B. RESULTS FROM BLUEGILL CHECK STANDARDS

Check standards were made up of pooled homogenates on the dates indicated, and analyzed along with sample homogenates with each cholinesterase analysis. BG√ required dilution with each use; all other homogenates were used at the concentration at which they were stored.

First Check Standard, "BG√," made 5/4/95.
N = 29; Mean Vmax = 85.30 ± 6.825 (CV = 8.00)

Date	Mean VMAX	CV
5/5/95	84.74	4.627
6/7/95	103.5	1.955
6/7/95	103.4	1.052
5/11/96	77.39	6.532
5/11/96	77.14	4.583
6/15/96	91.46	2.521
6/15/96	87.11	5.248
6/15/96	83.18	8.284
6/15/96	85.65	6.820
6/16/96	91.10	4.183
6/16/96	84.99	6.724
6/26/96	81.16	7.587
6/26/96	80.71	3.983
7/1/96	86.87	3.940
7/1/96	86.87	6.763
7/1/96	74.95	3.099
7/2/96	89.40	1.891
7/2/96	87.96	3.500
7/2/96	91.85	4.752
7/2/96	84.43	7.019
7/2/96	87.82	4.685
7/2/96	87.93	1.251
7/3/96	75.82	6.933
7/3/96	85.55	6.149
7/3/96	79.78	1.992
7/9/96	80.92	5.148
7/9/96	80.33	8.923
7/9/96	80.07	4.733
7/9/96	81.61	8.814

Second Check Standard, "BG2," made 6/1/95
N = 25; Mean Vmax = 121.59 ± 6.78 (CV = 5.58)

Date	Mean VMAX	CV
6/1/95	118.7	3.530
6/1/95	119.3	3.174
6/1/95	108.1	2.786
6/7/95	133.7	5.744
6/30/95	122.3	5.617
6/30/95	126.6	3.987
7/7/95	112.5	5.692
4/26/96	127.7	2.890
4/26/96	118.7	7.336
5/11/96	124.2	2.893
5/11/96	115.5	4.396
5/11/96	120.5	2.635
5/24/96	113.1	3.967
5/24/96	124.7	5.579
5/24/96	121.2	9.165
5/24/96	127.1	2.248
5/24/96	121.0	2.853
5/28/96	131.1	3.770
5/28/96	125.6	5.792
5/28/96	105.6	5.946
5/28/96	128.5	2.550
7/24/96	124.7	5.579
7/24/96	121.2	9.165
7/24/96	127.1	2.248
7/24/96	121.0	2.853

Third Check Standard, "BG3," made 7/3/96.
N = 23; Mean Vmax = 91.55 ± 5.65 (CV = 6.17)

Date	Mean VMAX	CV
7/3/96	91.54	4.122
7/9/96	97.39	2.588
7/9/96	90.88	6.383
7/15/96	91.07	3.506
7/15/96	96.57	4.288
7/15/96	95.99	4.611
7/15/96	80.46	5.031
7/15/96	90.44	5.086
7/28/96	88.42	7.244
7/28/96	95.72	1.544
7/28/96	88.52	2.975
7/28/96	89.81	9.733
7/28/96	88.36	0.964
7/24/96	84.88	4.321
7/24/96	78.53	4.245
8/3/96	100.5	6.702
8/3/96	98.03	8.430
8/22/96	92.97	9.484
8/22/96	92.30	5.478
8/22/96	94.87	6.748
8/22/96	91.09	8.438
8/22/96	100.4	1.484
8/22/96	86.93	3.122

Fourth Check Standard, "BG-4," made 8/22/96.
N = 11; Mean Vmax = 128.5 ± 5.28 (CV = 4.11)

Date	Mean VMAX	CV
8/22/96	133.5	0.308
8/22/96	134.8	0.996
9/21/96	133.8	5.595
9/21/96	120.5	8.188
9/21/96	133.3	3.228
9/22/96	122.4	4.680
9/22/96	124.6	1.656
9/29/96	128.1	9.507
9/29/96	129.0	5.291
9/29/96	122.4	6.084
9/29/96	131.5	3.772

APPENDIX C. STANDARD OPERATING PROCEDURE FOR CHIRONOMID CULTURES

I. Introduction

Chironomid larvae of the species, *Chironomus riparius* and *C. tentans* are used in toxicity testing. Proper culturing techniques assure an adequate supply of animals as well as reliable responses of larvae to toxicants. Larvae subjected to inadequate nourishment or other stressors may be overly sensitive to toxicants.

Eggs for *C. riparius* and *C. tentans* may be obtained from the Midwest Science Center (MSC) in Columbia, MO. MSC also has a standard operating procedure (MSC, 1993) for care of cultures. This procedure is derived from that one, but is modified for continuous cultures (i.e., neither eggs nor adults are routinely removed to new start new cultures).

II. Materials

A. Culture Tanks

1. 40-L aquaria.

2. Aeration source (e.g., air pump)--necessary because of high BOD.

3. Paper towels or cotton balls or other suitable material for substrate.

4. Screened lid to contain adults.

B. Food

1. TetraFin® goldfish food flakes (Tetra Sales, USA, Blacksburg, VA).

2. Green algae from culture or dried algae such as Spirulina (available from Argent Chemical Laboratories, Redmond, WA).

3. Blender for making suspension of TetraFin®. Standard household model is sufficient.

III. Feeding

1. TetraFin® tropical fish food flakes. Mix up a suspension of 30 g/L using a blender, refrigerate for up to 3 weeks. Add to cultures at a rate that will give 0.01 mg TetraFin®/mL culture water per day, to prevent food build-up and subsequent fungal growth.

2. Green algae from culture or suspension of dried algae. Feed twice a week to supplement TetraFin.

IV. Water Quality

1. Weekly test and record: temperature, DO, pH, alkalinity, total ammonia nitrogen, nitrite nitrogen.

2. Change water every other week. Partial water changes of at least half of the water in the tank are adequate. Watch for cloudiness of water, or for total ammonia nitrogen concentration above 0.02 mg/L. If either of these occur, increase frequency of water changes.

APPENDIX D. RESULTS FROM CHIRONOMID CHECK STANDARDS

Check standards were made up of pooled homogenates on the dates indicated, and analyzed along with sample homogenates with each cholinesterase analysis.

First Check Standard, "CR-1," made 6/28/95.
N = 35; Mean Vmax = 32.89 ± 2.25 (CV = 6.84)

Date	Mean Vmax	CV
7/4/95	33.72	0.975
7/4/95	32.38	2.970
7/4/95	32.80	1.951
7/28/95	31.46	10.57
7/28/95	34.95	3.441
7/28/95	34.37	4.769
8/7/95	31.78	14.00
8/7/95	36.21	4.945
8/7/95	37.27	1.124
8/8/95	31.71	3.752
8/8/95	34.83	6.142
8/8/95	37.52	18.12
8/31/95	32.17	4.420
8/31/95	31.94	8.180
8/31/95	30.31	1.712
8/31/95	32.19	1.065
8/31/95	36.18	9.017
8/31/95	34.52	9.919
9/4/95	33.18	7.004
9/4/95	32.63	7.783
9/4/95	36.11	6.572
9/4/95	34.88	7.385
9/7/95	33.99	3.524
9/7/95	31.71	3.131
9/7/95	32.04	1.283
9/7/95	30.78	8.150
9/8/95	29.62	1.816
9/8/95	29.28	4.341
9/8/95	29.26	5.723
9/8/95	29.22	10.23
9/13/95	33.82	5.272
9/13/95	30.15	5.181
9/14/95	31.81	1.374
9/14/95	32.73	3.698
9/14/95	33.67	8.239

Second Check Standard, "RIPAR-1," made 9/7/95.
N = 40 ; Mean Vmax = 35.25 ± 2.84 (CV = 8.07)

Date	Mean Vmax	CV
9/7/95	38.51	3.792
9/8/95	36.07	4.117
9/8/95	37.55	8.228
9/13/95	34.03	7.930
9/13/95	36.92	8.655
9/14/95	34.80	2.330
9/14/95	39.64	3.850
9/14/95	33.53	3.191
9/15/95	40.18	8.288
9/15/95	42.49	3.951
9/15/95	41.37	14.29
9/17/95	30.30	13.70
9/17/95	34.59	5.469
9/17/95	35.92	13.18
9/17/95	36.30	5.671
9/19/95	36.65	8.645
9/19/95	37.07	13.47
9/22/95	37.53	12.19
9/22/95	37.47	8.641
9/22/95	33.76	7.044
9/22/95	37.29	4.912
9/22/95	36.53	3.204
9/22/95	35.96	8.419
9/22/95	33.53	7.805
9/28/95	34.06	7.686
9/28/95	32.84	4.241
9/28/95	34.55	3.224
10/05/95	33.57	3.680
10/05/95	30.51	9.697
10/05/95	35.05	2.994
10/06/95	32.68	12.12
10/06/95	32.00	3.790
10/06/95	29.97	3.719
10/06/95	33.32	0.601
10/06/95	35.94	2.081
10/07/95	32.13	3.514
10/07/95	32.57	6.656
10/07/95	34.11	4.275
10/07/95	33.27	0.979
10/07/95	35.56	8.194

Third Check Standard, "RIPAR-2," made 9/22/95.
N = 34; Mean Vmax = 20.63 ± 1.51 (CV = 7.32)

Date	Mean Vmax	CV
9/22/95	20.17	5.301
9/22/95	18.23	2.925
9/22/95	21.22	2.972
9/28/95	21.15	6.857
9/28/95	21.47	7.822
10/05/95	20.61	6.363
10/05/95	19.80	3.550
10/05/95	20.54	7.897
10/06/95	19.05	3.669
10/06/95	19.31	3.914
10/06/95	18.17	4.002
10/06/95	18.65	6.949
10/08/95	21.61	2.454
10/07/95	21.01	2.947
10/07/95	18.50	6.281
10/08/95	20.69	6.402
10/08/95	18.33	1.400
10/08/95	19.55	12.50
10/19/95	19.57	5.661
10/19/95	21.86	5.873
10/19/95	19.95	8.067
4/22/96	22.01	7.111
4/22/96	21.40	6.813
4/22/96	21.41	0.810
4/22/96	22.49	3.330
7/10/96	20.14	6.629
7/10/96	20.11	12.92
7/10/96	19.86	4.832
7/10/96	22.76	6.904
7/10/96	21.07	9.966
7/10/96	20.88	4.763
7/13/96	24.74	1.515
7/13/96	22.01	10.30
7/13/96	23.19	6.504

Fourth Check Standard, "RIPAR-3," made 7/13/96.
N = 28; Mean Vmax = 35.43 ± 1.60 (CV = 4.53)

Date	Mean Vmax	CV
7/13/96	35.90	2.447
7/13/96	32.72	1.811
8/7/96	35.17	3.497
8/7/96	33.71	9.454
8/7/96	36.01	4.768
8/7/96	36.21	7.408
8/7/96	36.42	2.000
8/7/96	34.76	7.648
8/14/96	35.35	5.903
8/14/96	37.14	4.660
8/14/96	37.59	3.819
8/14/96	36.03	5.834
8/14/96	35.88	4.205
8/14/96	36.11	6.816
8/14/96	38.38	3.934
8/14/96	36.48	9.921
8/14/96	33.63	5.241
8/14/96	34.54	5.142
8/14/96	38.60	4.274
8/27/96	36.28	5.225
8/27/96	33.99	6.733
8/27/96	33.53	7.068
8/27/96	36.50	7.544
8/27/96	36.73	2.511
8/29/96	33.97	2.602
8/29/96	33.04	7.141
8/29/96	34.09	3.700
8/29/96	33.17	7.209

Fifth Check Standard, "RIPAR-4," made 8/29/96.
N = 7; Mean Vmax = 25.45 ± 2.27 (CV = 8.91)

Date	Mean Vmax	CV
8/29/96	28.63	2.636
8/29/96	27.78	8.024
10/3/96	25.36	1.656
10/3/96	24.05	3.233
10/3/96	22.00	10.65
10/3/96	25.94	2.829
10/3/96	24.37	8.921

Fifth Check Standard, "RIPAR-4," following inadvertant storage for a few days at room temperature, then refreezing, 10/2/96.
N = 22; Mean Vmax = 12.83 ± 1.85 (CV = 14.39)

Date	Mean Vmax	CV
11/25/96	17.60	12.05
11/25/96	15.54	14.11
12/23/96	14.99	6.207
12/23/96	13.84	7.253
12/29/96	11.11	8.283
12/29/96	10.94	5.478
12/29/96	11.19	5.755
12/31/96	15.11	7.774
12/31/96	13.63	7.018
12/31/96	13.32	7.285
12/31/96	13.53	2.013
12/31/96	12.47	12.35
12/31/96	12.23	13.51
12/31/96	12.01	13.19
1/1/97	12.22	2.319
1/1/97	13.99	5.900
1/1/97	11.93	7.384
1/1/97	10.14	7.004
1/1/97	12.65	5.223
1/1/97	11.37	5.529
1/2/97	12.26	12.68
1/2/97	10.25	2.114

Sixth Check Standard, "RIPAR-5," made 11/25/96.
N = 34; Mean Vmax = 69.59 ± 2.85 (CV = 4.09)

Date	Mean Vmax	CV
11/25/96	70.53	4.349
11/26/96	69.61	4.140
11/26/96	69.51	1.631
11/26/96	66.51	7.954
11/26/96	66.44	5.669
11/26/96	67.94	5.684
11/26/96	71.00	4.482
11/26/96	70.43	6.122
11/30/96	65.56	5.963
11/30/96	73.27	5.467
11/30/96	72.35	1.403
12/1/96	72.77	7.712
12/1/96	74.44	5.458
12/1/96	74.70	3.013
12/1/96	72.10	4.549
12/7/96	74.11	6.876
12/7/96	72.99	6.905
12/7/96	65.87	10.66
12/7/96	70.65	8.648
12/7/96	67.54	10.82
12/23/96	68.56	2.309
12/23/96	69.16	4.089
12/29/96	68.63	2.636
12/29/96	69.42	1.391
12/29/96	68.99	1.808
12/29/96	67.17	9.639
1/2/97	67.63	7.344
1/2/97	63.58	4.840
1/2/97	66.86	2.515
1/2/97	65.91	8.242
1/2/97	70.85	4.115
1/2/97	69.15	5.797
1/2/97	68.44	9.584

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